

CHAPTER 27

Flaviviruses

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The Flaviviridae are a family of at least 66 viruses, 29 (44%) of which have been associated with human disease, including the most important arthropod-borne viral afflictions of humankind—dengue, yellow fever, and Japanese encephalitis. In addition, eight flaviviruses cause disease in domestic or wild animals of economic importance.

HISTORY

Yellow fever virus, the family prototype (*L. flavus* yellow), was the first filterable agent shown to cause a human disease and the first virus proved to be trans-

missible by an arthropod vector. These discoveries occurred on the threshold of the twentieth century, some 350 years after the first clinical description of the disease. Yellow fever virus was the first flavivirus to be isolated (in 1927) and cultivated *in vitro* (in 1932). During the first decade of this century, dengue virus was also shown to be a filterable virus transmitted by arthropods, but it was not isolated until 1943.

A number of diseases characterized by meningo-encephalitis were recognized as nosologic entities during the nineteenth and twentieth centuries and were later proved to be caused by flaviviruses. Among these are louping ill (a disease of sheep, recognized in Scotland since 1807), Japanese encephalitis (Japan, 1873), and Australian X disease (now known as *Murray Valley encephalitis*, Australia, 1917). Between 1931 and 1937 the viruses responsible for louping ill, St. Louis

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encephalitis, Japanese encephalitis, and tick-borne encephalitis were isolated. Common features of these viruses included neurotropism and arthropod transmission, but initially they were believed to be totally distinct agents. In the late 1930s and early 1940s, relationships were demonstrated by neutralization and complement fixation between Japanese encephalitis, St. Louis encephalitis, and West Nile viruses. With the advent of the hemagglutination-inhibition test, which defined the broadest spectrum of antigenic relatedness, Casals and Brown (54) were able to separate the flaviviruses (group B arboviruses) from alphaviruses (group A viruses) and to define the cross-reactions among a set of 10 flaviviruses. As the flavivirus family grew with continued isolations of new agents from wild vertebrates and arthropods, so did the complexity of their serologic taxonomy (53). In the last decade alone (1978–1988), 24 new flaviviruses have been recognized.

Notwithstanding the lack of a serologic relationship, the group A and B arboviruses were originally linked on the basis of their mode of transmission and physicochemical characteristics into a single family, the *Togaviridae*, encompassing small RNA viruses with lipid envelopes and cubic nucleocapsid symmetry. As knowledge of the morphogenesis, biochemistry, and replication strategy of the flaviviruses expanded, it was clear by 1984 that their differences when compared to other togaviruses were sufficiently great to place them in a separate family (351). Great strides have been made in the molecular characterization of flavivirus structure and replication in the last 5 years (see Chapter 25), including publication of the full gene sequence of several viruses.

INFECTIOUS AGENTS

Physical and Chemical Properties

Flavivirus morphology, morphogenesis, chemical composition, and genome structure are described in detail in Chapter 25. Here we focus on the characteristics of virions which relate either to interactions with the environment or to laboratory manipulations in antigen and vaccine preparation. In the next section, the chemical and physical properties of gene products interacting with antibody and immunologically specified cells are described.

Flavivirus particles consist of a spherical ribonucleoprotein core surrounded by a lipoprotein envelope with small surface projections. Envelope lipids constitute approximately 17% of the virion dry weight (333) and are derived from the host-cell lipids. Lipases and lipid solvents disrupt flavivirus particles. Inactivation by chloroform and sodium deoxycholate provides a useful preliminary step in identifying flavivi-

rus (and other enveloped arboviruses). Acetone, which is often used to extract flavivirus antigens from infected mouse brain tissue, also destroys infectivity, whereas addition of sucrose partially preserves it. Hydrolysis by beta-propiolactone is an effective inactivating procedure that retains flavivirus antigenic reactivity to a greater extent than does formalin or phenol.

Flavivirions contain three structural proteins: a nucleocapsid or core protein (C; MW 14 kd), a nonglycosylated membrane protein (M; 7 kd), and an envelope protein (E; 50 kd) which is usually glycosylated (333,334). The M and E proteins are closely associated with the lipid envelope. The E protein is the major component of the virion surface projections observed by electron microscopy; it contains the important antigenic determinants subserving hemagglutination and neutralization and thus induces immunological responses in the infected host. E protein determinants are involved in the binding of virions to cell receptors and probably play a role in intraendosomal fusion at low pH. These protein constituents are sensitive to enzymatic digestion with trypsin, chymotrypsin, and papain, which render the virus noninfectious but preserve certain antigenic reactivities.

Detergents and proteases have been used to characterize the structure of flaviviruses and to isolate immunologically reactive subunits (319,334). Nonionic detergents, such as Triton-X, solubilize the entire envelope, releasing M and E proteins, whereas sodium deoxycholate appears to remove only E, leaving M associated with the nucleocapsid. Protease treatment showed that a portion of the E glycoprotein is located within the lipid bilayer (151). Recent analysis of the primary structure of the flavivirus glycoprotein has confirmed the presence of a hydrophobic membrane anchor region at the carboxyl terminus of the E protein molecule (243).

The flaviviral envelope protects the genome from cellular nucleases, and naked nucleocapsids released by detergent treatment are degraded by ribonuclease. Flavivirus infectivity and hemagglutinin are optimally stable at pH 8.4–8.8 (169). Sensitivity to acid pH (and to bile and enzymes) generally precludes infection by the oral route. Tick-borne encephalitis may, however, be acquired by ingestion of infected milk, and, in contrast to other flaviviruses, the tick-borne agents are relatively resistant to hydrogen ions (253). The structural and molecular bases for this difference have yet to be defined.

Flaviviruses are rapidly inactivated at high temperature. At 50°C, 50% of infectivity is lost in 10 min. As a practical measure, total inactivation of virus suspended in blood or other protein solutions occurs within 30 min at 56°C. Low temperatures preserve infectivity, with stability being greatest at –60°C or below.

Aerosols present a hazard of laboratory infection. St. Louis encephalitis virus was stable for 6 hr in aerosol suspension at room temperature and 23–80% humidity (169).

Flaviviruses are inactivated by ultraviolet light, gamma-irradiation, and disinfectants, including 3–8% formaldehyde, 2% glutaraldehyde, 2–3% hydrogen peroxide, 500–5,000 ppm available chlorine, alcohol, 1% iodine, and phenol iodophors. The tick-borne viruses appear to be relatively more resistant to these measures than mosquito-borne agents.

Antigenic Composition and Determinants

Antigenic Classification

Flaviviruses contain antigens reactive in binding assays (immunofluorescence and enzyme or radionuclide-based immunoassays), complement-fixation (CF) assays, hemagglutination-inhibition (HI) and neutralization (N). The flaviviruses were originally grouped on the basis of cross-reactivities in the HI test performed with polyclonal antisera (54). The HI test and binding assays detect group-reactive determinants, whereas the CF test is intermediate in specificity, and the N test is relatively type-specific. The N test is used to distinguish individual viruses in the family and to define subgroups of closely related viruses.

On the basis of cross-neutralization using polyclonal, hyperimmune antisera, the flaviviruses have been divided into a number of antigenic complexes. De Madrid and Porterfield (73) classified 36 flaviviruses in seven complexes or subgroups, whereas six remaining viruses were antigenically distinct. A more recent analysis (48) of 66 flaviviruses defined eight antigenic subgroups encompassing 49 viruses, leaving 17 others unassigned (Table 1). This antigenic classification conforms to major biological and epidemiological characteristics of the flaviviruses.

Monoclonal Antibody Analysis

In the 1950s and 1960s, Clarke (60,61) analyzed the antigenic structure of flaviviruses by antibody absorption, providing evidence that group, complex, and type-specific determinants existed on the hemagglutinin and setting the stage for studies at the molecular level.

The use of monoclonal antibodies has greatly extended our understanding of flavivirus antigenic interrelationships and structure (for excellent reviews of this subject, see refs. 149 and 276). Serological analyses have demonstrated the presence of flavivirus group, complex, and type-specific antigenic determinants, generally confirming the relationships shown

TABLE 1. *Flavivirus antigenic complexes defined by close relationships in cross-neutralization tests with polyclonal antisera^a*

Principal vector	Antigenic complex	Viruses
Tick	Tick-borne encephalitis	(<i>Russian spring-summer encephalitis</i> , <i>Central European encephalitis</i> ^{b,c}), <i>Omsk hemorrhagic fever</i> , <i>louping-ill</i> , <i>Kyasanur forest disease</i> , (<i>Langat</i> , <i>Phnom-Penh bat</i> [*] , <i>Carey Island</i> [*]), <i>Negishi</i> , <i>Powassan</i> , <i>Karshi</i> , <i>Royal Farm</i>
	Tyulenyi	<i>Tyulenyi</i> , <i>Saumaurez Reef</i> , <i>Meaban</i>
Mosquito	Japanese encephalitis	<i>Japanese encephalitis</i> , <i>St. Louis encephalitis</i> , <i>Murray Valley encephalitis</i> , <i>West Nile</i> , <i>Kunjin</i> , <i>Usutu</i> , <i>Kokobera</i> , <i>Stratford</i> , <i>Alfuy</i> , <i>Koutango</i>
	Ntaya	<i>Ntaya</i> , (<i>Tembusu</i> , <i>Yokose</i>), (<i>Israel turkey meningoencephalitis</i> , <i>Bagaza</i>)
	Uganda S	<i>Uganda S</i> , <i>Banzi</i> , <i>Boutoui</i> , <i>Edge Hill</i>
	Dengue	<i>Dengue 1</i> , <i>dengue 2</i> , <i>dengue 3</i> , <i>dengue 4</i>
None ^d	Rio Bravo	<i>Rio Bravo</i> , <i>Entebbe bat</i> , <i>Dakar bat</i> , <i>Bukalusa bat</i> , <i>Apoi</i> , <i>Saboya</i>
	Modoc	<i>Modoc</i> , <i>Cowbone Ridge</i> , <i>Jutiapa</i> , <i>Sal Vieja</i> , <i>San Perlita</i>

^a Modified from ref. 48. The antigenic complexes correspond roughly to vector associations, implying evolutionary origin. Seventeen other flaviviruses are sufficiently distinct to preclude inclusion in these complexes.

^b Parentheses indicate viruses more closely related to each other than to other members of the complex.

^c Italics indicate viruses pathogenic for humans and/or domesticated animals.

^d Viruses transmitted directly between vertebrate hosts, principally bats and rodents. Two members of the TBE complex (indicated by asterisk) may have this mode of spread.

with polyclonal antisera. Monoclonal antibodies have also been found with subcomplex, subtype, strain, and even substrain specificity. With polyclonal antisera, specificity at these levels has been difficult to demonstrate, requiring special techniques such as HI antibody absorption with heterologous antigens or kinetic neutralization. Although monoclonal antibodies reveal a wide spectrum of specificities in both binding and functional assays, binding assays (such as immunofluorescence) generally have not revealed antigenic differences at the strain or substrain level, whereas such differences may be evident in HI or N tests. Nonoverlapping type- and strain-specific neu-



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tralization epitopes have been found, as expected from the relative specificity of neutralization with polyclonal antisera.

Studies with monoclonal antibodies against yellow fever, Japanese encephalitis, St. Louis encephalitis, dengue, and tick-borne encephalitis have also uncovered antigenic relationships at the epitope level which do not correspond to cross-reactivities revealed by polyclonal antisera, thus complicating the study of flavivirus variation and biology. Monoclonal antibodies have been found which link different flavivirus antigenic complexes or which reveal previously unsuspected relationships between members of the same antigenic complex. For example, among members of the dengue complex, subcomplex specificities were demonstrated for dengue 1 and 4 and dengue 2 and 3 (155). Relationships were also revealed between dengue and members of other antigenic complexes (e.g., Tembusu virus). Moreover, monoclonal antibodies may show functional reactivity with heterologous, but not homologous, virus strains. For example, monoclonal antibodies prepared against yellow fever 17D virus neutralized the parental Asibi strain but not 17D (296). To complicate matters further, binding and functional assays with the same monoclonal antibody may show completely different cross-reactivity patterns. Finally, monoclonal antibody analyses of variation in antigenic reactivity between strains of individual flaviviruses have not generally allowed classification by geographic origin. An exception is dengue type 2 virus, for which antigenic differences were detected that correspond to geographic variation defined by RNA fingerprinting (230).

Binding of certain monoclonal antibodies may be enhanced in the presence of other antibodies (153). The synergistic effect, which may be unidirectional or bidirectional, is the result of a conformational change in epitope presentation mediated by the first antibody;

this change, in turn, increases binding activity of the second antibody. The patterns of cooperation between monoclonal antibodies have been useful in mapping flavivirus epitopes (149) and in the construction of diagnostic tests (231).

Topological maps of flavivirus E glycoprotein epitopes have been constructed on the basis of (a) competitive binding with monoclonal antibodies, (b) serological specificity, and (c) biologic activity. Comprehensive studies have been performed with tick-borne encephalitis (150), dengue (157), St. Louis encephalitis (275), yellow fever (50), and Murray Valley encephalitis (145). Multiple epitopes with group, complex, subcomplex, and type specificity have been found, forming linkage groups as well as nonoverlapping antigenic domains. For example, an antigenic model of the yellow fever 17D E glycoprotein showed five nonoverlapping antigenic domains, including one linkage group of six overlapping epitopes with flavivirus group reactivity (Fig. 1). Vaccine-specific, substrain-specific, type-specific, and intercomplex-specific epitopes were located in separate domains. Functional activity was associated with all but the vaccine-specific domain, and heterogeneous functions (HI, N, protective capacity) were present within individual domains. These findings demonstrate that individual antigenic domains on the E glycoprotein spike contain epitopes with different functional activities and serological specificities and that epitopes subserving similar functions can be located in distinct antigenic domains.

Until recently, such antigenic models have not been correlated with sequence data or information about secondary and tertiary structure of the E protein. The latter are undoubtedly important, since monoclonal antibody analyses indicate that individual epitopes are often constituted by distant amino acid sequences brought into proximity by tertiary folding. The struc-

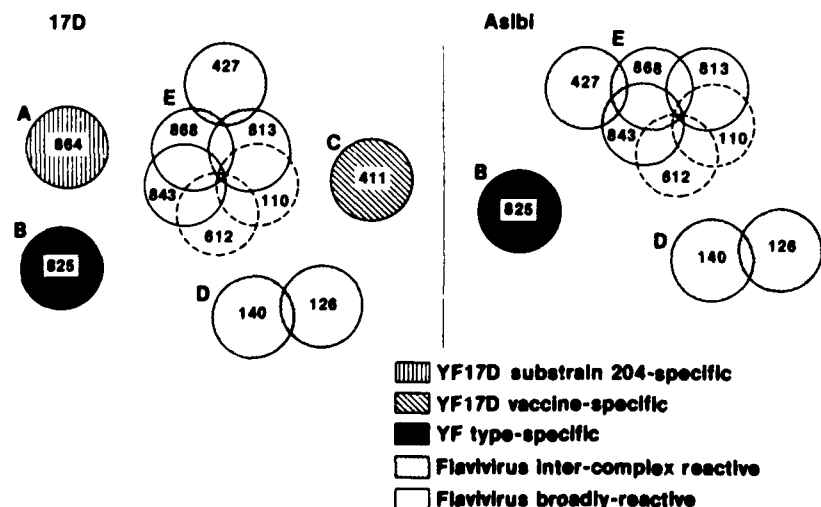


FIG. 1. Model of spatial arrangement of epitopes on the E glycoprotein of yellow fever 17D vaccine virus and its virulent parent, Asibi. In both strains, there is a domain (designated E) of overlapping antigenic sites defined by competitive binding of monoclonal antibodies and incorporating mainly flavivirus-group-reactive determinants. Topographically distinct substrain-specific and 17D-strain-specific epitopes are defined, as well as a yellow-fever-type-specific determinant (B), and epitopes are shared with one or more members of other flavivirus antigenic complexes. (Modified from ref. 50 with permission.)

ture of the E protein of West Nile and Central European encephalitis (CEE) viruses has been elucidated by analysis of the disulfide bridges in the E glycoprotein (200,243). In the CEE model, the major domain (domain A, Fig. 2) begins at amino acid residue 60 at the N-terminus of the E glycoprotein; it includes (a) a region stabilized by three disulfide bonds extending to residue 121 and (b) a juxtaposed loop representing residues 200–250. Domain A contains strongly hydrophilic regions and antigenic epitopes involved in neu-

tralization and hemagglutination. Sequence analyses of neutralization escape variants and immune-reactive fragments obtained by chemical degradation have localized antigenic determinants within the E protein structure (191,200). Within the A domain, a shared neutralization epitope of both yellow fever (191) and CEE (200) viruses has been mapped at position 71 in the N-terminal (A) domain (Fig. 2). Important antigenic sites involved in neutralization and hemagglutination also lie in the B domain (200,204), a separate

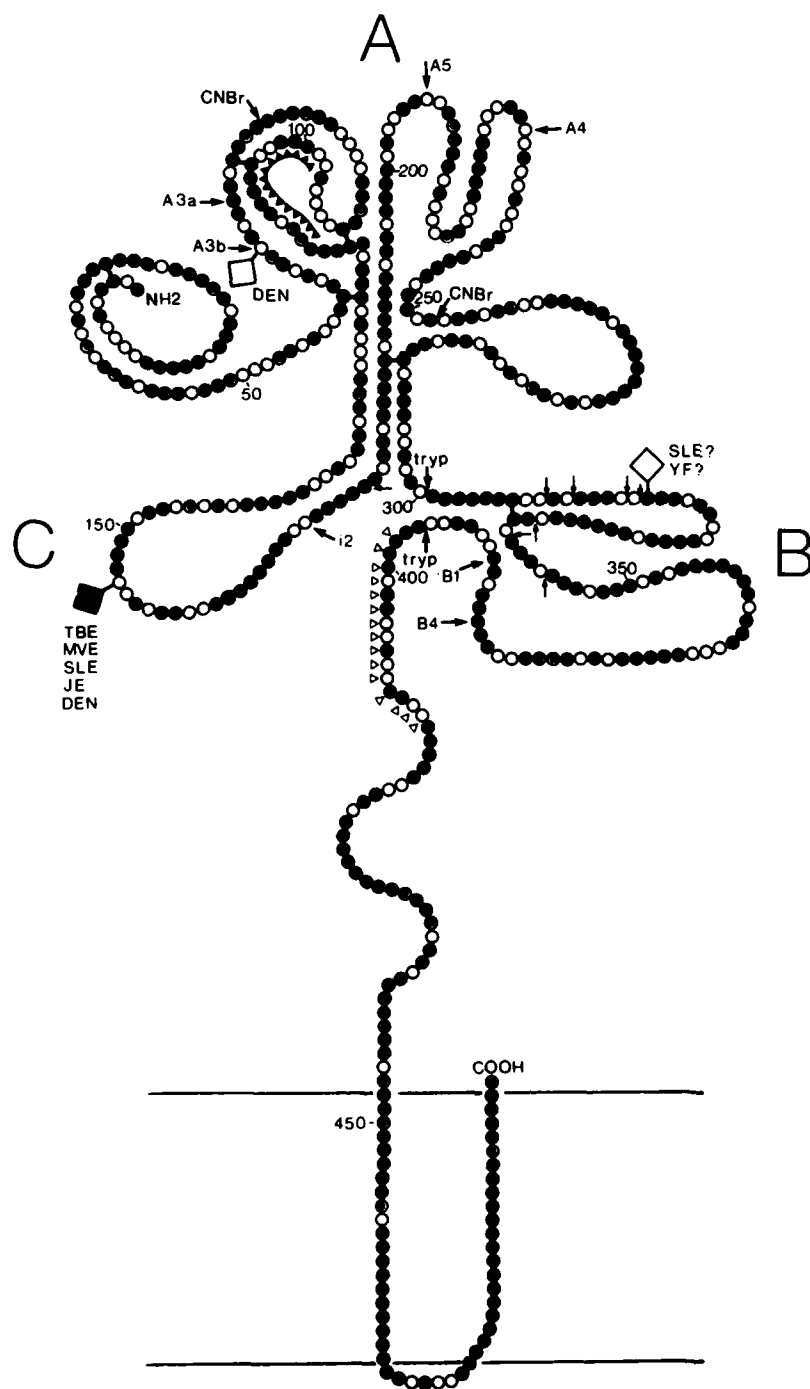


FIG. 2. Model of flavivirus envelope (E) protein, indicating structure determined by folding of the polypeptide chain into discontinuous antigenic domains A, B, and C. Unfilled circles represent hydrophilic amino acids, filled circles represent hydrophobic amino acids, and circles with dots indicate intermediate amino acids. Cysteine residues forming disulfide bridges are connected by solid lines. Arrows indicate protease cleavage sites that liberate immunoreactive fragments. Arrows with monoclonal antibody designations show sites identified by sequencing neutralization escape mutants. [A3a corresponds also to the N-escape variant of yellow fever at position 71 (191).] A row of filled triangles indicates the putative site of virus-cell receptor binding. Glycosylation sites for various flaviviruses are shown as diamonds. (From ref. 200 with permission.)

structural region representing residues 301–395 in the CEE model and structurally dependent upon a disulfide bridge. The C domain is the most variable and contains epitopes with little serologic cross-reactivity or biological activity. Residues 98–111 are highly conserved among flaviviruses, are located as a hydrophobic area between disulfide bonds in domain A, and may subserve binding of virions to cell-surface receptors or fusion of virion envelope to endosomal membrane. At the carboxyl terminus of the E protein, there are hydrophobic segments that span the lipid membrane of the virion envelope and serve to anchor the glycoprotein spike.

Protective and Cross-Protective Immunity

Recovery from flavivirus infection or active immunization results in long-lasting protection against homologous challenge. Many investigators have also shown varying degrees of cross-protection between members of individual antigenic complexes and even between members of different complexes (reviewed in ref. 341). These experiments raised hopes that immunization with one or more flavivirus antigens might confer protection against heterologous viruses. Indeed, cross-protection appears to play a role in nature; for example, prior dengue exposure decreases morbidity associated with St. Louis and Japanese encephalitis (29,140). However, despite considerable effort, cross-protective vaccine schedules have not come into use.

The molecular basis for homologous and heterologous protection has been partially elucidated. In passive protection studies, monoclonal antibodies with high *in vitro* neutralizing activity (and often with type-specificity) confer protection at low dose levels, leading to the concept of a "critical neutralization site" (33,276). This determinant has been located at amino acid position 71 in the flavivirus E protein (191). However, both protective and N activities are also associated with complex- and group-reactive epitopes. Antibodies against these epitopes generally must be given at high doses to demonstrate protection, although this is not invariably so. Non-neutralizing protective epitopes have also been defined. Antibodies to these epitopes may have complex, subcomplex, and group specificity, and they may confer heterologous cross-protection. For example, a group-reactive non-neutralizing dengue antibody (4G2) provides efficient protection against yellow fever challenge (33). The ability of non-neutralizing antibodies to protect was not associated with (a) avidity, (b) spatial proximity to the critical neutralizing epitope, or (c) C-dependent lysis of virus-infected cells. Although they showed no neutralization in Vero cells, the protective antibodies in-

hibited virus replication in a neural cell line, indicating a possible mechanism for protection.

Immunization with the NS1 protein of yellow fever and dengue has been shown to confer protection against homologous challenge (297,298). Despite the presence of complex-reactive epitopes on dengue NS1, cross-protection in mice was not observed.

Cell-mediated immune responses may play an important role in viral clearance. Dengue serotype-specific delayed-type hypersensitivity responses have been described (248). Murine L3T4⁺ T-cell clones demonstrated extensive cross-reactivity among members of the Japanese encephalitis (JE) complex (339).

Antibody-Dependent Enhancement

Antibody-dependent enhancement (ADE) of flavivirus replication in Fc-receptor-bearing peripheral blood monocytes and macrophage-like cell lines has been demonstrated *in vitro* with a number of flaviviruses, including dengue, yellow fever, Wesselsbron, West Nile, and tick-borne encephalitis viruses (134,137). Increased adsorption of virus particles to host-cell plasma membrane (mediated by subneutralizing antibodies), as well as increased efficiency of virion internalization, has been shown to increase viral replication five- to sixfold in monocyte- or macrophage-like cells (111,112). A second type of enhancement dependent upon the C3 complement receptor has also been described (51). Attempts to elucidate the epitopes responsible for ADE by use of monoclonal antibodies have yielded conflicting results. Depending upon the virus and cell system used, monoclonal antibodies with both type-specific and group-reactive specificity have mediated enhanced virus replication (139). The possible role of ADE in the immunopathogenesis of dengue hemorrhagic fever is discussed below.

Nonstructural Antigens

A number of nonstructural virus-specified antigens are expressed in flavivirus-infected cells, and some are exposed on the plasma membrane, rendering them susceptible to antibody and cell-mediated immune elimination. Among the nonstructural antigens, the NS1 glycoprotein assumes great biological importance and is both associated with infected cell membranes and released as a soluble complement-fixing antigen (307). Antibodies to NS1 have been demonstrated in human serum (92) and have been shown to cause complement-mediated lysis of infected cells *in vitro*. In addition, both passive transfer of NS1 antibodies and active im-

munization with NS1 antigen have protected animals against flavivirus challenge (298).

NS1 monoclonal antibodies afford protection against (a) virus challenge by C-mediated cytolysis and (b) elimination of infected cells (298). Antibodies to NS1 do not bind to virus particles and thus would not be involved in antibody-mediated enhancement of virus replication in mononuclear cells (a presumed pathogenic mechanism in dengue hemorrhagic fever). For this reason, NS1 protein is an attractive candidate for subunit vaccine development. Both serotype-specific and cross-reactive antigenic determinants have been found within NS1. NS1 epitopes of dengue 2 virus have been topologically mapped and were arranged in six distinct, but overlapping, sites (156).

Virus Virulence and Biological Characteristics: Molecular Basis

Strains of yellow fever virus (19,79), St. Louis encephalitis (SLE) (225), and Japanese encephalitis virus (161,162) vary in neurovirulence or neuroinvasiveness for mice. The molecular basis for these differences remains elusive, although strain comparisons have demonstrated differences in RNA oligonucleotide fingerprints and nucleotide sequences. In the case of SLE virus, strains with low neurovirulence phenotype were shown to have in common a unique nucleotide substitution within a highly conserved sequence in the 5'-terminal noncoding region (37). This region may be an important signal sequence for flavivirus replication (271).

Current evidence regarding the molecular basis for virulence comes from comparative studies of attenuated 17D yellow fever vaccine and its virulent, viscerotropic parent, the Asibi strain. The vaccine and parent viruses are separated by approximately 240 passages in mouse and chick cells. The viruses have minor differences in their RNA fingerprints and in their E and NS1 proteins by gel electrophoresis (76). Differences in the topographical distribution of biologically functional epitopes on 17D and Asibi viruses have been described (Fig. 1). The 17D E protein contains at least two epitopes absent from Asibi. At the sequence level, there was overall divergence of 68 nucleotides, resulting in 32 amino acid changes (131). Twelve (a considerable proportion) of these changes were in the E structural protein. Since the E glycoprotein is involved in interactions with host-cell receptors, one or more of these changes might affect tropism and replication.

Virus-Cell Interactions

A major change in infected cells is proliferation and hypertrophy of rough endoplasmic reticular mem-

branes within which virus particles accumulate. Host-cell macromolecular synthesis is not markedly decreased until late in flavivirus infection, when cytopathic effects (CPEs) appear. As discussed further below, many arthropod and vertebrate cultured cells do not exhibit CPEs. In other cell-virus pairings, infection progresses to CPEs within an interval as short as 18 hr or as long as 5-7 days. At the ultramicroscopic level, cellular pathologic changes include mitochondrial damage, fragmentation of reticular membranes, formation of distended vacuoles and inclusion bodies, increase in lysosomal bodies, and rarefaction of cytoplasm (235). Activities of lysosomal enzymes increase in infected tissues. At the microscopic level, susceptible vertebrate cells, such as HeLa, BHK-21, porcine kidney, and primary chick or duck embryo display cell rounding, shrinkage, pyknosis of nuclei, and dislodgement from the growth surface (Fig. 3). Cell fusion and syncytial formation (polykaryocytosis) have been observed in BHK-21 cells.

Unlike vertebrate cells, arthropod cells infected with some flaviviruses are capable of massive proliferation and hypertrophy of cytoplasmic membranes, as well as very great production of virus, without sustaining cytopathologic changes. Infection of mosquito cells with certain flaviviruses may result in CPEs characterized by syncytium formation (250). In contrast to mosquito cell cultures, those derived from ticks do not demonstrate CPEs after infection with tick-borne flaviviruses.

In general, mosquito-borne viruses replicate in mosquito cell culture, and a few (such as SLE and West Nile which have been isolated from ticks in nature) will also grow in tick cell cultures. Tick-borne flaviviruses replicate in tick cell cultures, but not consistently in mosquito cells.

The antigenic reactivity of some flaviviruses is altered by replication in mosquito cells. For example, dengue and Kunjin viruses lose hemagglutinating activity on passage in *Aedes albopictus* cells (238).

Propagation and Assay in Cell Culture

Flaviviruses produce CPEs and plaque formation in a variety of primary and continuous cell cultures derived from human, monkey, rodent, swine, and avian tissues (reviewed in ref. 169). Cell cultures of reptilian, amphibian, and arthropod origin also support replication with or without CPEs or plaques. Virus yields and titers, grade of CPE, plaque size and quality, and rate of growth vary with the specific virus and host cell (see discussion of individual viruses). In general, the BHK-21 (baby hamster kidney), SW-13 (human adrenal carcinoma), PS (porcine kidney), *Aedes* mosquito cells, and primary chick and duck embryo cells

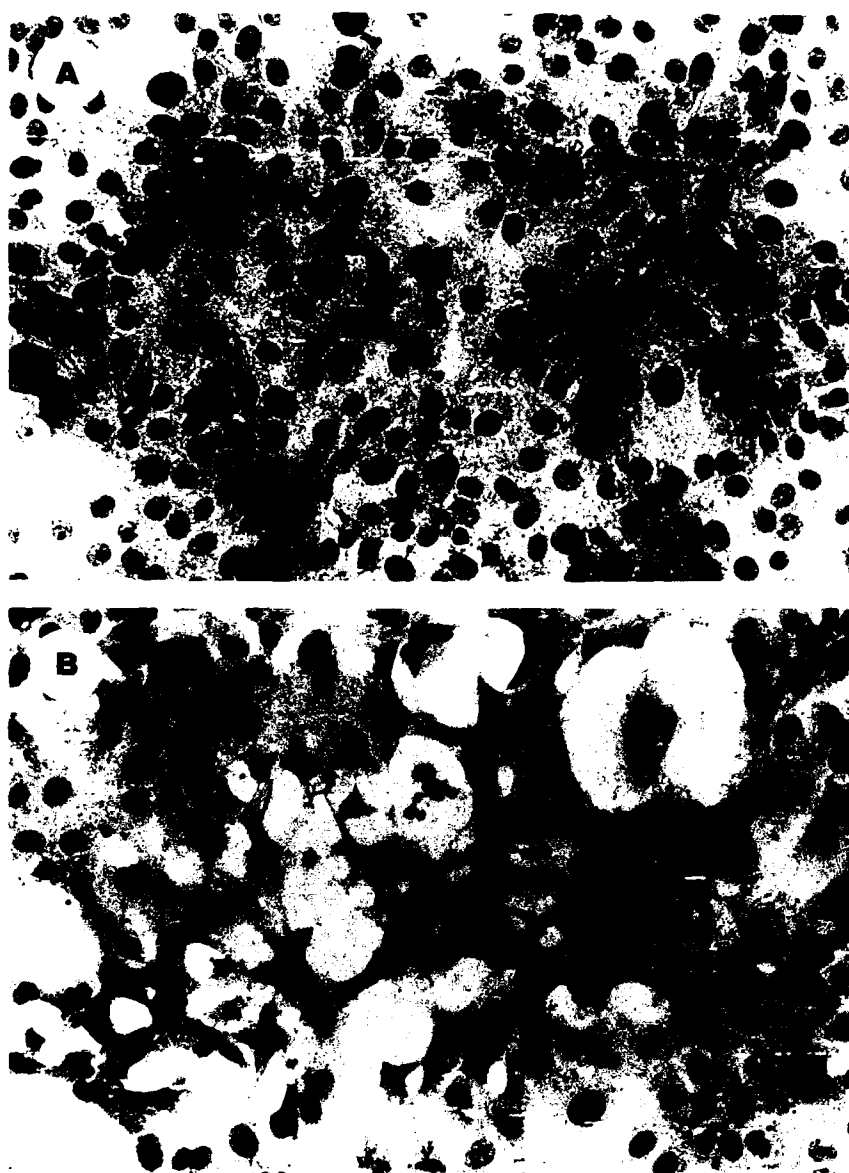


FIG. 3. Cytopathic effects of St. Louis encephalitis virus in Vero cell culture. **A:** Normal culture, Mayer's hematoxylin, $\times 312.5$. **B:** Culture after 5 days incubation, showing focal cytopathic effects, shrinkage, and rounding of cells, pyknotic nuclei, and appearance of holes in the cell monolayer.

produce the highest virus yields, in the range of 9–10 d.e./ml. In comparison with alphaviruses, the latent period of flavivirus growth is relatively long, and growth is slow. At high multiplicity of infection in BHK-21 cells, SLE virus has a latent period of 11 hr and reaches peak titer at 28 hr. Plaques appear after 4–6 days; double overlay procedures are often required for optimal plaque assays in mammalian cell cultures. Addition of neutral red to agar overlay may enhance plaque size. Assay of dengue virus infectivity requires special techniques (see below).

In addition to observation of CPEs or plaques, flavivirus growth in cell culture may be measured by immunofluorescent staining or detection of antigen in supernatant fluids by immunoassay, CF, or hemagglutination. In the case of arthropod cells that

do not produce CPEs or plaques, these techniques are mandatory, or supernatant fluid must be passed to a susceptible host (mice or mammalian cell culture.)

Infection in Experimental Animals; Host Range

The susceptibility of experimental animals and host range vary and are considered in the discussion of individual viruses.

Newborn mice and hamsters inoculated intracerebrally are somewhat more sensitive for infectivity assay and primary isolation of many flaviviruses than are cell cultures; the slight advantage in sensitivity is, however, offset by the expense and inefficiency of *in vivo* assays. For other flaviviruses, including yellow

fever and dengue, cell cultures provide more sensitive assay systems. In general, intrathoracic or intracerebral inoculation of live mosquito adults or larvae provides the most sensitive system for assay of mosquito-borne flaviviruses.

PATHOGENESIS AND PATHOLOGY

The greatest body of information about flavivirus pathogenesis is derived from experiments on mice and other laboratory rodents. These animals provide a reasonably good model of flavivirus encephalitis but not of other syndromes associated with human flavivirus infection (i.e., fever–arthralgia–rash and hemorrhagic fever). Viruses that produce these syndromes in humans, including dengue and yellow fever, cause encephalitic infections in laboratory rodents. The pathogenesis of dengue and yellow fever is discussed in their respective sections. The universal neurotropism of flaviviruses in rodents and even in arthropod vectors (in which brain and ganglia are major sites of replication) reflects evolutionary conservation of viral polypeptide structures involved in receptor interactions and of cell membrane molecules which subserve virus–receptor interactions.

Three patterns of pathogenesis have been described in flaviviral encephalitis (222,236): (i) fatal encephalitis, usually preceded by early viremia and extensive extraneural replication; (ii) subclinical encephalitis, usually preceded by low viremia, late establishment of brain infection, and clearance with minimal destructive pathology; and (iii) inapparent infection, characterized by trace viremia, limited extraneural replication, and no neuroinvasion.

Virus and Host-Specified Factors Influencing Pathogenesis

The course and outcome of infection is influenced by both virus- and host-specified factors. High dose and intracerebral or intranasal routes of virus infection predispose to fatal encephalitis. Virus strains may differ in neuroinvasiveness and/or neurovirulence.

Among host factors influencing pathogenesis, the most important are age, sex, genetic susceptibility, and preexisting infection or immunity to heterologous agents. Neonatal animals are more susceptible to lethal encephalitis than older animals. Neonatal animals inoculated by the peripheral route are susceptible until 3–4 weeks of age, when resistance develops, but they may remain susceptible to lethal encephalitis when inoculated intracerebrally. In contrast to experimentally infected mice, humans exposed during infancy or childhood to some flaviviruses (e.g., SLE) usually experience mild infections; moreover, susceptibility to

encephalitis increases with advancing age, with the elderly being most severely affected. The mechanisms underlying the increasing susceptibility with age are not known, but they may include the presence of underlying diseases that impair immune function or reduce the effectiveness of the blood–brain barrier.

Sexually mature female mice demonstrate increased resistance to some flavivirus infections (7). Sex differences in susceptibility of humans (as opposed to exposure to infected vectors) have not been demonstrated, except possibly in the case of dengue hemorrhagic fever, which preferentially affects female children.

Genetic determinants play a central role in the pathogenesis of flavivirus infections. Studies by Webster (348) and Sabin (288) showed that resistance of non-immune mice to flavivirus infection was determined by a single autosomal dominant allele. The resistance allele was incorporated into susceptible C3H inbred mice to yield two histocompatible lines (C3H/He and C3H/RV) that differ in susceptibility to flaviviruses (121). West Nile virus yields in brains of resistant (C3H/RV) mice were significantly lower than in susceptible mice, but there were no differences in interferon or humoral antibody responsiveness between the two mouse strains (114). *In vitro* studies with cells derived from the two mouse strains indicated that (a) macrophages from resistant mice do not support flavivirus replication as well as macrophages from the susceptible strain and (b) the lower virus yields observed in cells of RV mice were due to greater production of defective interfering virus particles (71). In the case of Banzi virus infection of RV mice, genetic resistance did not appear to depend on lack of permissiveness of tissues to virus replication (164) but, instead, had an immunological basis (26,165). Virologic mechanisms may also operate in determining resistance in this model, however. Increased interfering virus found in lymphoid tissues of RV mice may contribute to survival (305).

Concurrent infections with unrelated agents may enhance flavivirus neuroinvasion (reviewed in ref. 222), presumably by disturbing the blood–brain barrier. This mechanism has been reported in mice doubly infected with Japanese encephalitis, herpesvirus, *Trichinella*, and visceral larva migrans; a similar phenomenon has been postulated to occur in humans with neurocysticercosis (299).

Extraneural Infection and Routes of Neuroinvasion

A general scheme for the dissemination of flaviviruses in the host is shown in Fig. 4. After inoculation into the skin, the virus replicates in local tissues and regional lymph nodes. Virus is then carried via lym-

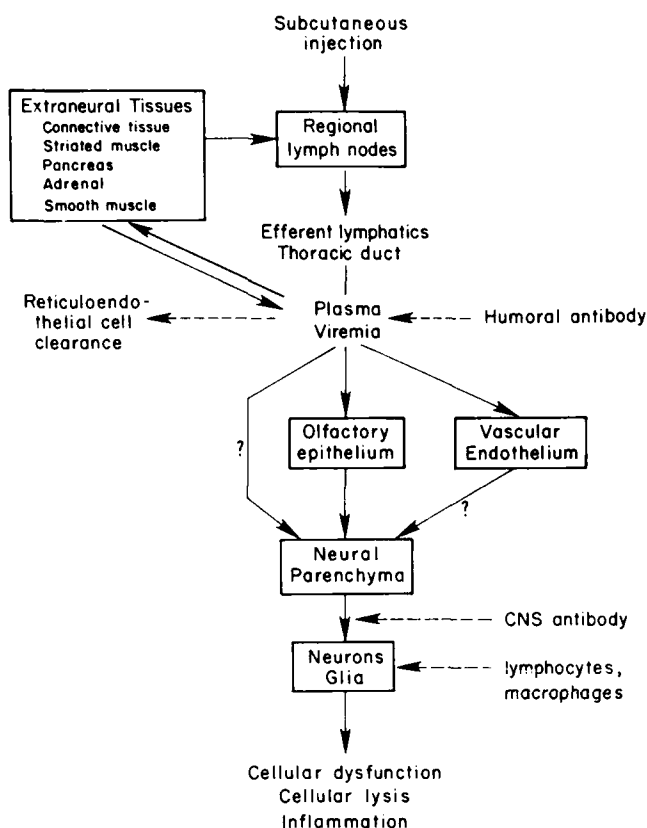


FIG. 4. General scheme of the sequential development of flavivirus infection. *Boxes* indicate sites of virus replication, and *dashed arrows* show immune defense mechanisms. (Modified from ref. 236 with permission.)

phatics to the thoracic duct and into the bloodstream (199). This primary viremia seeds extraneural tissues, which, in turn, support further viral replication and serve as a source for release of virus into the circulation.

The viremia level is modulated by the rate of clearance by macrophages, and it is terminated by the appearance of humoral antibodies, usually by approximately 1 week after infection. Major extraneural sites of flavivirus replication include connective tissue, skeletal muscle and myocardium, smooth muscle, lymphoreticular tissues, and endocrine and exocrine glands.

In baby hamsters infected with SLE and Rocio viruses, pancreas and heart were the most severely affected organs (144). Virus particles within secretory granules of exocrine and endocrine areas of the pancreas were released by exocytosis. Myocardial necrosis with productive viral infection of myocytes was a prominent finding. Correlations are possible between this experimental model and pathogenesis in clinical hosts. Infection of goat mammary glands, followed by secretion of virus in goat milk, is an important mode of spread of tick-borne encephalitis viruses. Interstitial

myocarditis has been reported in West Nile and Japanese encephalitis of humans and horses, and pancreatitis has been associated with human West Nile virus infection.

Investigation of experimental flavivirus encephalitis in mice has demonstrated a relationship between level of viremia, development of brain infection, and multisite appearance of viral antigen in nervous tissue (5), supporting the concept of hematogenous spread to the central nervous system (CNS) (167). The mechanism by which flavivirus particles cross the blood-brain barrier remains uncertain. The ability of these viruses to replicate in vascular endothelial cells suggests that they may "grow across" capillaries in the brain. However, viral antigen has been found only rarely in endothelial cells of brain capillaries.

The olfactory tract has long been recognized as (a) an alternative pathway to the CNS and (b) an important mode of spread following aerosol exposure. In an experimental model, mice and hamsters inoculated by the peripheral route with SLE virus developed low-level or undetectable viremias similar to those occurring in clinical hosts. This resulted in early infection of olfactory neurons (unprotected by blood-brain barrier) and subsequent axonal transport of virions to the olfactory lobe of the brain (226). It is not known whether this pathway operates in humans, but a postmortem study of JE patients indicated the hematogenous, rather than the olfactory, route of neuroinvasion (168). Once in the CNS, virus spreads rapidly.

Neuronal centers vary in susceptibility. In the mouse the hippocampal formation is particularly sensitive; in monkeys and humans the thalamus, substantia nigra, and cerebellum are most vulnerable (236,269).

Pathological Changes

Pathological changes include: (a) neuronal and glial damage caused directly by viral injury and characterized by central chromatolysis, cytoplasmic eosinophilia and cell shrinkage, and neuronophagia; (b) inflammation, including perivascular infiltration of small lymphocytes, plasma cells, and macrophages (Fig. 5); and (c) cellular nodule formation composed of activated microglia and mononuclear cells (Fig. 6). In monkeys and hamsters infected with tick-borne viruses, astrocyte proliferation and hypertrophy appear as a late phenomenon (367).

Residual neurological deficits, electroencephalographic changes, and psychiatric disturbances frequently persist after recovery from acute encephalitis. Pathological lesions in cases with neurological residua 12–67 years after recovery from acute JE were characterized by small areas of rarification (neuronal loss),

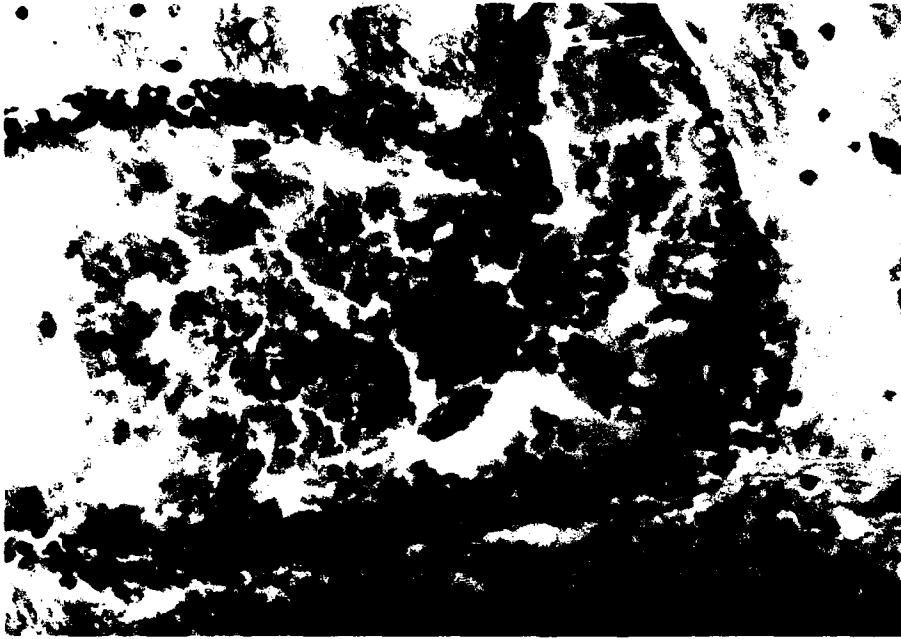


FIG. 5. Perivascular cuff of small lymphocytes around a vein in the hippocampus of a fatal human case of St. Louis encephalitis. Hematoxylin and eosin. $\times 400$. (Courtesy of M. G. Reyes.)

surrounded by dense microglial scarring, and were distributed in areas typically affected during the acute phase. In experimental animals, changes in behavior and learning ability have been documented (see the section entitled "Persistent and Congenital Infection," below).

Persistent and Congenital Infection

Subacute and chronic forms of encephalitis have been described in animals and humans, especially in association with tick-borne flaviviruses. Hamsters and

monkeys develop progressive neurological degeneration with astrocytic proliferation, perivascular granulomata, and neuronal vacuolation. Chronic infection of neural or lymphoreticular tissues has been reported in monkeys with tick-borne encephalitis and West Nile viruses and in mice with Kyasanur forest disease (reviewed in ref. 222). Virus isolates from persistently infected animals may exhibit phenotypic changes such as (a) loss of hemagglutinin, (b) reduced neurovirulence, and (c) temperature sensitivity similar to that associated with persistent infection of cell cultures. Studies of such viruses at the molecular level would be instructive.

In Asia, JE virus is an important cause of epizootic abortion and stillbirth in swine. JE virus has also been isolated from brain, liver, and placental tissues of aborted human fetuses (58). Mice infected with JE virus show a high incidence of stillbirth and congenital malformations when inoculated 9–16 days before parturition. Mice have been shown to transmit JE virus to offspring of consecutive pregnancies, and immune suppression during pregnancy has been postulated to play a role in establishment of persistent maternal infection (206,207). These considerations advise caution in the development and testing of live vaccines for neurotropic flaviviruses.

Immune Response

Studies of antibody responses in human cases of flaviviral encephalitis indicate that: (a) Persons with inapparent infections do not develop local CNS antibody



FIG. 6. Glial nodule in the substantia nigra from a fatal human case of St. Louis encephalitis. Hematoxylin and eosin. $\times 400$. (Courtesy of M. G. Reyes.)

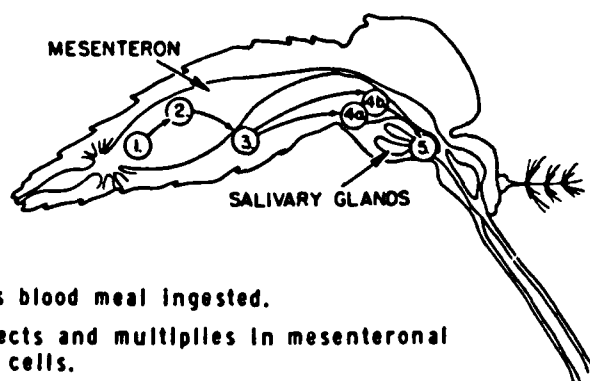
and thus have not sustained brain infection; and (b) during the acute phase of infection, persons with fatal encephalitis have absent, low, or delayed levels of virus-specific IgM and IgG in serum and cerebrospinal fluid, whereas patients with nonfatal encephalitis have vigorous systemic and local CNS antibody responses (45). These observations illustrate the dynamics of the "race" between humoral immune response, extra-neural replication, and neuroinvasion.

Both humoral antibody and cellular immune responses are responsible for viral clearance and recovery from infection. Whereas passively transferred antibodies can abort experimental flavivirus encephalitis even when given after neuroinvasion, transfer of immune spleen cells may not be effective at this stage. Perivascular and parenchymal inflammatory cells are immunologically specific and of hematogenous origin. Johnson et al. (168) studied the cellular components of CNS inflammation in fatal human cases of JE. At the time of death (3–9 days after onset), approximately 30–40% of cells in perivascular cuffs had T-cell markers, but only 5–10% were suppressor/cytotoxic cells. In severe or lethal infections characterized by high-titer viral growth and rapid accumulation of antigen in the critical target tissues, inflammatory responses may enhance lesions and accelerate death. Antibody-mediated early death in mice infected with yellow fever virus has been attributed to complement-mediated cytotoxicity of infected cells (115).

ARTHROPOD INFECTION

Biological transmission of flaviviruses by arthropods depends upon the following: ingestion of a blood

meal containing virus; infection of epithelial cells lining the mesenteron (midgut); escape of virus from the midgut epithelium into the hemocoel; infection of the salivary gland; and, finally, secretion of virus in saliva during refeeding on a susceptible vertebrate host (Fig. 7). Many flaviviruses exhibit a high degree of specificity in their ability to infect and be transmitted by individual insect or tick species (or even strains of individual species). Vector competence is under genetic control, with the susceptibility of the midgut epithelium being the primary determinant (for reviews, see refs. 143 and 336). In a susceptible arthropod, a sufficient concentration of virus must be ingested to exceed the mesenteron infection threshold. The time interval between the ingestion of an infectious blood meal and the salivary secretion of virus (extrinsic incubation period) must not exceed the life span of the arthropod and is an important, temperature-dependent factor determining the rate of virus transmission in nature. Flaviviruses generally do not have a direct pathogenic effect on vectors; however, in the case of vertical transmission of some agents in *Aedes* mosquitoes, a prolongation of development time from ova to pupa has been noted, suggesting a deleterious effect (326). Vertical transmission from female arthropod to her progeny is an important mechanism for overwinter survival of certain flaviviruses. Flaviviruses infect the genital tract of female mosquitoes so that the virus may enter the fully developed egg through the micropyle at the time of fertilization/oviposition (281). As opposed to true transovarial transmission (infection of the egg at the time of development in the ovary), this mechanism allows infection of mature ova during the first ovarian cycle after feeding on



- ① Infectious blood meal ingested.
- ② Virus infects and multiplies in mesenteron epithelial cells.
- ③ Virus released (escapes) from mesenteron epithelial cells.
- ④ a. Virus infects salivary glands after secondary amplification in other cells/tissues.
b. Virus infects salivary glands without secondary amplification in other cells/tissues.
- ⑤ Virus released from salivary gland epithelial cells and is transmitted by feeding.

FIG. 7. Steps required for flavivirus infection and transmission by an arthropod. (From ref. 143 with permission.)

a viremic host. Venereal transmission of flaviviruses from male to female mosquitoes has also been demonstrated (282).

FLAVIVIRUSES ASSOCIATED PRIMARILY WITH THE ENCEPHALITIS SYNDROME

St. Louis Encephalitis

The history of St. Louis encephalitis (SLE) has been reviewed in detail by Chamberlain (56). The disease was first recognized in 1932, when an outbreak occurred in Paris, Illinois. The following year there was a large epidemic in St. Louis and Kansas City, Missouri, during which the virus was isolated from human brain. *Culex pipiens* mosquitoes were suspected to transmit the infection in this outbreak on epidemiological grounds, but over 20 years would pass before this species was the principal vector in the east-central United States. During the early 1940s the disease was recognized in the Pacific coast states, where the virus was isolated from *Culex tarsalis*. Since 1933 there have been numerous outbreaks involving the western United States, Texas, the Ohio-Mississippi Valley, and Florida. The largest of these occurred in 1975, with nearly 2,000 recognized cases. Epidemic activity in the past decade, as well as major advances in research on the ecology of SLE, has recently been reviewed (229).

Infectious Agent

SLE virus is a member of the Japanese encephalitis antigenic subgroup. Antigenic and structural properties of the virus have been described (169,333), and its nucleotide sequence has been partially elucidated (332). The organization of the genome is similar to all other flaviviruses. As expected, a higher order of sequence homology (approximately 65%) was found with members of the Japanese encephalitis antigenic complex than with yellow fever or dengue (approximately 40%). SLE virus produces CPE and plaques in a wide variety of cell cultures (reviewed in ref. 169). High yields of virus (8–9 dex), high grades of CPE, and high plaque titers are obtained in continuous cell lines of hamster (BHK-21), monkey kidney (Vero, LLC-MK₂, MA-104), and porcine kidney (PS) origin and in primary cultures of chick and duck embryo. Mosquito cell cultures support growth with or without CPE. Infectivity titers in cell cultures are comparable to those in suckling mice.

Infant mice and hamsters are highly susceptible to lethal infection by the intracerebral and peripheral routes. With increasing age, these hosts develop resistance to peripheral challenge but remain susceptible to intracerebral inoculation. Virus strains vary considerably in virulence for mice and may be classified as

highly virulent, avirulent, or of intermediate virulence on the basis of the intraperitoneal/intracerebral LD₅₀ ratio (225). Mouse virulence correlates with pathogenicity for rhesus monkeys inoculated intracerebrally and with the capacity of virus strains to induce viremia in the house sparrow (31). Chickens develop viremia without disease; newly hatched chicks have the highest titers. Young rats inoculated intracerebrally are partially susceptible, and survivors may develop cataracts (142). Guinea pigs, older rats, and rabbits develop antibody but not disease or consistent viremia. SLE virus infects the chorioallantoic membranes of chick embryos.

Pathogenesis and Pathology

The pathogenesis in experimental animals has been described above. The neuropathology associated with human disease is reviewed by Gardner and Reyes (104). Principal lesions include neuronophagia, cellular nodules, and perivascular cuffing, most severely affecting substantia nigra, thalamus, and hypothalamus.

Clinical Features

Three clinical syndromes are described: encephalitis, aseptic meningitis, and febrile headache (Table 2). The severity of illness increases with advancing age, and persons over 60 years have the highest frequency of encephalitis. The incubation period varies between 4 and 21 days. Onset is characterized by generalized

TABLE 2. Clinical syndromes caused by infection with flaviviruses associated with CNS disease^a

Encephalitis ^b (including meningoencephalitis and encephalomyelitis)
Acute febrile illness (oral temperature 100°F, 37.8°C)
One or more signs in either of the following categories:
Altered level of consciousness (confusion, disorientation, delirium, lethargy, stupor, coma)
Objective signs of neurologic dysfunction (convulsion, cranial nerve palsy, dysarthria, rigidity, paresis, paralysis, abnormal reflexes, tremor, etc.)
Aseptic meningitis ^b
Acute febrile illness
Sign(s) of meningeal irritation (stiff neck with or without positive Kernig's or Brudzinski's sign)
No objective signs of neurologic dysfunction
Febrile headache ^b
Acute febrile illness
Headache (may also have other systemic symptoms, e.g., nausea or vomiting)
No signs of meningeal irritation or neurologic dysfunction

^a From ref. 36 with permission.

^b CSF pleocytosis present in patients with encephalitis and aseptic meningitis; it may also be found in patients with the syndrome of febrile headache.

malaise, fever, chilliness, headache, drowsiness, anorexia, nausea, myalgia, and sore throat or cough, followed in 1–4 days by the acute or subacute appearance of meningeal and neurologic signs. Early urinary tract symptoms (frequency, urgency, dysuria) occur in nearly one-fourth of the patients (262). There is no pathognomonic profile of neurologic findings. Altered level of consciousness, abnormal reflexes, tremor, and signs of thalamic, brain-stem, and cerebellar dysfunction (nystagmus, myoclonus, ataxia) are the most prominent findings. Cranial nerve involvement may occur, particularly lower motor neuron n. VII deficit. Approximately 10% of patients have convulsions—a poor prognostic sign. Approximately 50% of patients with fatal infections die within 1 week of onset, and 80% die within 2 weeks of onset. The case-fatality rate increases with age, from 2% in young adults to over 22% in the elderly. The disease may be complicated by bronchopneumonia, bacterial septicemia, pulmonary embolism, or gastrointestinal hemorrhage. Underlying hypertensive and arteriosclerotic disease, diabetes, and chronic alcoholism predispose to severe infection and fatal outcome.

A number of clinical laboratory abnormalities have been described, including an elevated peripheral white blood cell count and increased serum transaminase, creatine phosphokinase, and aldolase levels. The urinalysis may show pyuria, microscopic hematuria, and proteinuria, and there may be an elevated blood urea nitrogen level (262). The cerebrospinal fluid (CSF) shows moderate pleocytosis (≤ 500 cells/mm³), mainly lymphocytes, although polymorphonuclear cells may predominate early in the infection. The CSF protein may be elevated (usually 45–100 mg%, rarely as high as 500 mg%). There are a few reports of hypoglycorrhachia. Hyponatremia and hypo-osmolality occur in up to one-third of the patients as a result of inappropriate secretion of antidiuretic hormone (353). Elevated plasma 17-hydroxycorticosteroids and loss of the normal diurnal pattern of glucocorticoid secretion indicate reaction to stress (85). A disproportionately high cerebral perfusion in relation to metabolic demands has been found, indicating a disturbance in cerebral autoregulation of blood flow, which was unrelated to changes in sensorium of acutely ill patients. Radio-nuclide brain scans and computed tomography have been normal. The electroencephalogram shows diffuse generalized slowing and amorphous generalized delta wave activity (36).

A period of prolonged convalescence occurs in 30–50% of cases, characterized by aesthenia, irritability, tremulousness, sleeplessness, depression, memory loss, and headaches, lasting up to 3 years. Approximately 20% of these patients have symptoms persisting for longer periods, including gait and speech disturbances, sensorimotor impairment, psychoneurotic

complaints, and tremors (93). Old age and severity of acute illness appear to predispose to these sequelae.

Differential Diagnosis

The patient's age, season of the year, place of residence and exposure, and information about the occurrence and serodiagnosis of similar cases in the community are of paramount importance in the differential diagnosis. In the individual case, it is essential to rule out treatable bacterial, mycobacterial, spirochetal, and fungal infections as well as herpes encephalitis. Because SLE often strikes the elderly, it has occasionally been misdiagnosed as stroke.

Laboratory Diagnosis

Virus isolations from serum or CSF are very unusual, and testing is not profitable. In over one-half of fatal cases, virus may be recovered by intracerebral inoculation of suckling mice with suspensions of brain tissues; occasional isolates have been made from liver, spleen, lung, and kidney (49). SLE viral antigen has been demonstrated by careful immunofluorescence examination of brain frozen sections (269). Flavivirus-like particles have been found by electron microscopy, and SLE antigen has been found by immunofluorescence in urine sediment (197).

Specific diagnosis usually relies on serological tests on appropriately timed acute and convalescent samples. The HI test detects mainly group-reactive antigens and is thus a useful screening procedure. Antibody titers increase rapidly during the first week after onset. In primary infection, titers to SLE antigen are usually higher than to heterologous antigens. In some areas of the southern United States, cross-reactions due to immunity to dengue virus confuse the diagnosis in older individuals. CF antibodies appear during the second week and peak at 3–4 weeks after onset. Because CF antibody titers then fall off to low levels by 9–12 months, a diagnosis can often be made by demonstrating a fourfold or greater fall in titer between early and late convalescent sera. The presence of CF antibody in a single serum sample is presumptive evidence of a recent infection. However, 20% of patients with confirmed SLE virus infections fail to develop detectable CF antibodies (49). Cross-reactions in the CF test are less than by HI. The neutralization test is most specific. Antibodies appear during the first week and persist for many years, usually for a lifetime.

Local production of IgM antibodies in the CNS (105) provides a potential means of rapid and early diagnosis. Demonstration of IgM antibodies in CSF by enzyme-linked immunosorbent assay (ELISA) appears

also applicable to the diagnosis of SLE as early as 3–5 days after onset.

The IgM-capture ELISA has replaced classic serologic methods in many laboratories. IgM antibodies in serum appear within the first 4 days after onset, peak at 7–14 days, and decline thereafter, generally reaching extinction by 60 days (228). Presence of IgM antibody in a single serum is presumptive evidence of recent infection. However, because IgM antibody may persist for up to a year in up to one-fourth of patients, demonstration of a decrease in antibody titer between paired sera is preferable. IgA antibodies parallel those in the IgM class (335).

Treatment

Neither antiviral chemotherapeutic agents nor interferon have been evaluated for therapy of SLE. Treatment is supportive and consists of good general management and nursing care, especially in the semicomatose and comatose patient.

Hyponatremia secondary to inappropriate antidiuretic hormone (ADH) secretion is managed with water restriction. Marked, progressive elevations in intracranial pressure have not been documented in SLE, but this possibility should be considered in severely ill patients with deepening coma and loss of brain-stem reflexes. Anticonvulsant therapy may be required.

Epidemiology

Morbidity and Mortality

The epidemiology of SLE has recently been reviewed (219,229). Since the inception of nationwide surveillance in 1955, nearly 5,000 cases of SLE have been officially reported in the United States. Horses and other domesticated animals do not develop clinical signs of infection. The disease occurs in epidemic form at approximately 10-year intervals (Fig. 8). Outbreaks have involved up to 1,815 notified cases. Endemic transmission occurs during interepidemic intervals, with small numbers of notified cases (<50 per year). Attack rates in localities affected by epidemics have ranged from 1 to 800 per 100,000 population. The disease appears in July, with the peak incidence in August and September, but outbreaks may occur later in the year at southern latitudes. SLE predominantly affects the Ohio–Mississippi Valley, eastern Texas, Florida, Kansas, Colorado, and California (Fig. 9). The vectors responsible for transmission vary regionally. In the Ohio–Mississippi basin and eastern Texas, the distribution of cases is urban–suburban, corresponding to high densities of the principal vectors, *Culex pipiens*

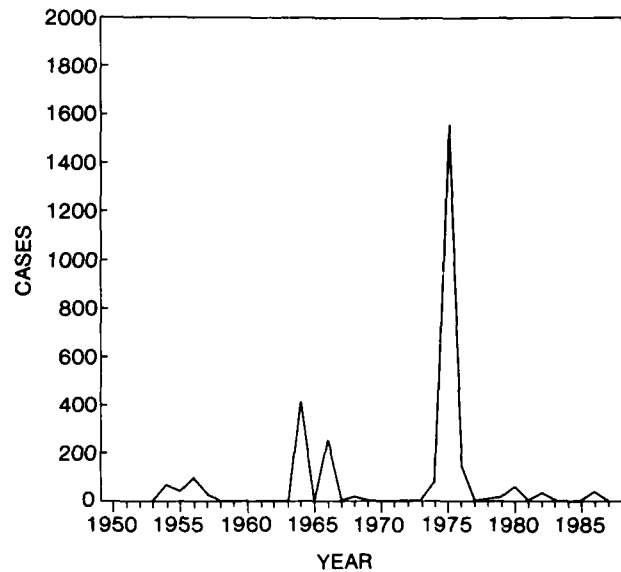


FIG. 8. Annual incidence of St. Louis encephalitis transmitted by *Culex pipiens* complex mosquitoes in the eastern United States; epidemic activity has reappeared at approximately 10-year intervals. (Modified from ref. 219 with permission.)

and *Culex quinquefasciatus*, which breed in polluted water (especially where poor sanitation exists). In Florida the tropical mosquito *Culex nigripalpus* is the epidemic vector. In the western states the principal vector, *Culex tarsalis*, breeds in irrigated or flooded dryland areas; its wide distribution leads to frequent human exposures in rural areas. *Culex tarsalis* is also the vector of western equine encephalitis virus, and transmission of this virus and of SLE are often concurrent. SLE occurs as a sporadic disease in many areas of tropical America (315). Outbreaks have occurred in Jamaica.

In the eastern and central United States the incidence of disease and the case-fatality rate are up to 40 times higher in individuals over 55 years than in younger persons. Although previously obscured by a high background of naturally acquired immunity in endemic areas of the western United States, the greater susceptibility of the elderly has become evident in the West. Altered behavioral patterns of the human population (use of air-conditioning and television) may be responsible for decreased exposure to mosquitoes, as well as a decline in infection and immunity, in these endemic areas (100).

In rural areas of the western states, where *Culex tarsalis* is the principal vector, cases in males outnumber those in females nearly 2:1; this is because of the greater opportunity of exposure of males working outdoors. A predominance of cases in females has been found in the central and eastern states, where exposure to the household mosquito vectors (*Culex pipiens*, *Culex quinquefasciatus*) is responsible for infection.

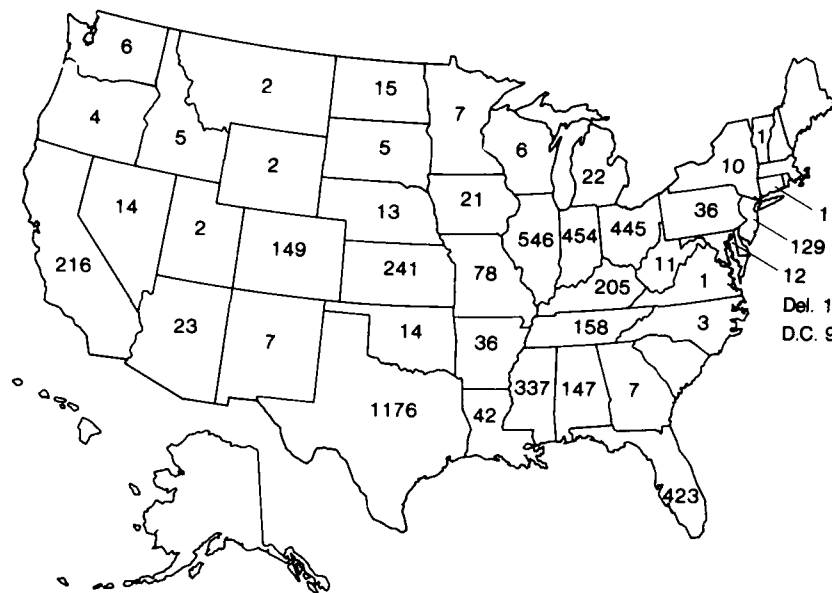


FIG. 9. Distribution of officially reported St. Louis encephalitis cases, by state, 1955–1986.

In most *Culex pipiens*-borne outbreaks in the southern United States, the incidence has been highest in black populations inhabiting lower socioeconomic areas of affected cities where environmental conditions favor breeding of the vector. In Florida, however, the black population was relatively spared during epidemics between 1959 and 1962. Elderly blacks had cross-protective antibodies to dengue virus (29).

Immunity

Surveys in several urban areas in the eastern United States have shown a 6% incidence of SLE virus infection during epidemics (159). A survey of urban and rural Indiana residents showed an overall seroprevalence of 3.6% and an estimated annual infection rate of 0.32% (120). The ratio of inapparent to apparent infection varies with age, from 806:1 in children to 85:1 in the elderly. No recent assessment of seroprevalence has been made in rural areas of the western United States.

Origin and Spread of Epidemics

Evidence indicates that SLE virus is maintained in local winter reservoirs in North America, but the possibility of annual reintroduction from warm regions of year-round transmission by migratory birds or bats cannot be excluded. The virus has been isolated from hibernating adult female *Culex pipiens* (14), and there is annual recrudescence of virus activity throughout much of North America. Vertical transmission of virus has been experimentally shown in *Culex pipiens*, *Culex*

quinquefasciatus, *Culex restuans*, and other species (reviewed in ref. 229). SLE virus has been isolated from the gizzard of a cowbird 38 days after experimental infection (57) and from the blood of bats for prolonged periods (321). Transplacental passage of virus in bats has also been demonstrated. Despite these observations, the mechanism(s) whereby SLE virus is maintained locally over the winter will require further elucidation.

Amplified transmission of the virus begins in the springtime and early summer, with the re-emergence and breeding of vector mosquitoes. If conditions are favorable, a rapid, cumulative, and progressive increase in the transmission cycle follows, involving the epidemic vectors. Wild passerine birds serve as the main viremic hosts in these cycles of transmission. A comprehensive review of the avian species involved is given by McLean and Bowen (215). If the rate of virus transmission between birds and mosquitoes is sufficiently high, humans and other mammals (horses, dogs, etc.) may be tangentially infected but do not serve as viremic hosts. Many factors influence the rate of spread of virus (Table 3) and determine the course and extent of epidemics (265).

Prevention and Control

At the present time, no vaccine against SLE is available. Reduction of vector populations remains the most widely used method for prevention and control of SLE epidemics. Surveillance programs focus on early detection of increased levels of virus activity by testing avian sera for antibodies or mosquitoes for virus.

TABLE 3. Some factors that affect rates of flavivirus transmission in nature^a

Virus
Strain differences in infectiousness for vectors and hosts
Vectors
Susceptibility to infection and efficiency of transmission ("vector competence")
Vector population density, population dynamics, and age structure
Longevity
Preference for biting host species
Distribution and dispersal (flight range)
Reservoir-hosts
Susceptibility to develop effective viremia
Host population density, population dynamics, and age structure
Active and passive immunity
Attractiveness to vectors
Distribution and dispersal
Clinical hosts (e.g., humans)
Population density
Acquired immunity
Exposure to vector bites

^a Modified from ref. 265.

Molecular Approaches

SLE virus strains exhibit extensive variability in their RNase T₁ oligonucleotide fingerprints (333). Strains can be classified by their geographic origin; moreover, within a given area, both genetic drift and introduction of other geographic types have been demonstrated over time. The technique thus provides a possible means of determining the origin and source of outbreaks. The presence of unique genetic variants in Florida (*Culex nigripalpus* cycle), the Ohio-Mississippi River basin (*Culex pipiens* and *quinquefasciatus*), and the western United States (*Culex tarsalis*) suggests that the virus is maintained in each area in local reservoirs. RNA fingerprints also correlate with biological (virulence) characteristics of strains. Comparison of the 5'-terminal nucleotide sequences of SLE strains of low virulence have shown a unique base substitution at position 16 (37), but the relationship of this change to biological markers remains uncertain.

Japanese Encephalitis

A disease resembling Japanese encephalitis (JE) was recognized in horses and humans as early as 1871. A severe epidemic occurred in Japan in 1924, and a filterable agent was extracted from human brain and passed to rabbits. In 1934, Hayashi reproduced the disease in intracerebrally inoculated monkeys. In 1935, the agent was recovered from the brain of a human in

Tokyo and was virologically and serologically established as the prototype (Nakayama) strain. The virus was first recovered from brain tissue of a sick horse in 1937. Mosquito transmission was suspected during the early 1930s; in 1938, Mitamura et al. reported isolation from *Culex tritaeniorhynchus*. Classic studies in Japan by Scherer et al. (40,293) established that pigs and birds were the principal viremic hosts and that *Culex tritaeniorhynchus* was responsible for transmission between these vertebrates and from them to humans.

Epidemics of JE recur in temperate areas of Asia and in the northern part of tropical Southeast Asia. In terms of morbidity and mortality, this disease is by far the most important of the arbovirus encephalitides (for a review, see ref. 337).

Infectious Agent

JE virus is the prototype of the JE antigenic complex. The complete nucleotide sequence of the JE viral genome has been determined (324), and JE viral antigens have been expressed in yeast (99) and bacteria (204). Antigenic variation has been shown by antibody-absorption HI, agar gel diffusion, CF, kinetic neutralization, antibody-absorption neutralization (244), and monoclonal antibody analysis (178), but no clear geographic classification has emerged. At least two immunotypes have been repeatedly distinguished: Nakayama (representing the prototype strain isolated from human brain in Japan in 1935) and JaGAr 01 (from *Culex* mosquitoes, Japan, 1959). Virus strains isolated in 1969-1970 were immunologically placed between these types (244). To further complicate matters, antigenic differences have been shown between substrains of Nakayama virus. The recognition of antigenic strain variation has led to altered strategies for vaccine preparation (see section entitled "Prevention and Control," below).

The virus replicates in a wide variety of primary and continuous cell cultures of hamster, porcine, chicken, monkey, and mosquito origin. Vero and LLC-MK₂ cells are useful for plaque assays (317).

Infant mice are highly susceptible to lethal infection by all routes of inoculation. Weanling mice succumb to intracerebral virus inoculation, but there is virus strain variation in pathogenicity by the intraperitoneal route. Hamsters and monkeys die after intracerebral inoculation but develop asymptomatic viremia after peripheral infection. Rabbits and guinea pigs have asymptomatic infections by all routes of inoculation. The virus is pathogenic for embryonated chicken eggs. It produces disease in horses and swine (see below).

Pathogenesis and Pathology

Considerable variation exists in neurovirulence and peripheral pathogenicity for mice among JE virus strains (161). Sites of replication and dissemination of virus in the mouse have been described by Huang and Wong (162) and are similar to those described earlier.

During the acute stage, congestion, edema, and small hemorrhages are found in the brain. Microscopic lesions include neuronal degeneration and necrosis, neuronophagia, glial nodules, and perivascular inflammation. These changes occur in gray matter and predominantly affect diencephalic, mesencephalic, and brain-stem structures. Destruction of cerebellar Purkinje cells may be prominent. A variety of pathological changes in extraneural tissues have also been noted, including hyperplasia of germinal centers of lymph nodes, enlargement of malpighian bodies in spleen, interstitial myocarditis, swelling and hyaline changes in hepatic Kupffer cells, pulmonary interalveolitis, and focal hemorrhages in the kidneys.

In one study of fatal human cases, JE viral antigen was localized to neurons, with no evidence for glial cell infection (168). The highest concentration of infected neurons was in thalamus and brain stem. Among inflammatory cells recruited into perivascular infiltrates, T cells predominated, but a minority were T suppressor/cytotoxic lymphocytes. Macrophages predominated among cells recruited into the brain parenchyma.

Dual human infections with JE and herpes simplex viruses have been described. In analogous experiments in mice, JE viral antigen was localized in herpesvirus-infected areas of the brain, suggesting that JE virus gained access to the CNS at sites of blood-brain barrier disruption caused by herpesvirus (146). A similar role has been implicated for *Toxocara canis* and *Trichinella* in experimental dual infections (69,251) and for *Taeniasis solium* (neurocysticercosis) in humans (299).

Transplacental infection in swine results in abortion and stillbirth; abortuses show encephalitic lesions. The virus also produces hypospermia and aspermia in boars (130). Histopathological changes include epididymitis, spermatogenic arrest, and inflammation of the tunica testis. Transplacental infection in humans has been documented (58), resulting in abortion and isolation of virus from fetuses. Pregnant mice inoculated intraperitoneally also transmit JE virus to the fetus, with subsequent abortion (206). A curious feature of this model is that infected mothers, when mated again after 6 months, transmitted virus to the second litter. Latent infections of pregnant mice could be reactivated by cyclophosphamide or subsequent pregnancy.

Clinical Features

The incubation period is 6–16 days. As for SLE, illness may be manifested by a febrile headache syndrome, aseptic meningitis, or encephalitis (81,106). In the full-blown encephalitic form, onset is rapid, beginning with a 2- to 4-day prodromal phase of headache, fever, chills, anorexia, nausea and vomiting, dizziness, and drowsiness. In children, abdominal pain and diarrhea may be prominent. These symptoms are followed by the appearance of nuchal rigidity, photophobia, altered states of consciousness, hyperexcitability, and varying objective neurological signs, including dull, mask-like facies, muscular rigidity, cranial nerve palsies, tremulous eye movements, coarse tremors of the extremities, involuntary movements, generalized and localized paresis, incoordination, and pathologic reflexes. Sensory deficits are rare. Paralysis of the upper extremities is more common than paralysis of the legs. Spinal cord involvement (lower motor neuron deficits) may occur, and a bulboparetic syndrome has been described. Convulsions are frequent in children but occur in less than 10% of adult patients. Severe hyperthermia may require specific countermeasures. Death occurs on the fifth to ninth day or during a more protracted course with cardiopulmonary complications. A poor prognosis is associated with respiratory dysfunction, positive Babinski's sign, frequent or prolonged seizures, prolonged fever, albuminuria, infectious virus in CSF, and low levels of IgM and IgM antibodies in serum and CSF (41).

The peripheral white blood cell (WBC) count is mildly elevated, with a relative neutropenia and a lymphopenia due to a decrease in T cells. Urinary tract symptoms are common during the acute phase of illness and may be accompanied by sterile pyuria and microscopic hematuria and albuminuria. The CSF pressure may be elevated; microscopic examination shows 10–500 (rarely up to 1,000) WBCs, predominantly lymphocytes. Cell counts fall gradually over 8–9 weeks. The CSF protein concentration is mildly elevated (50–100 mg%). The electroencephalogram (EEG) is abnormal, with decreased electrical activity, slowing, and dysrhythmia.

Neuropsychiatric sequelae occur in up to 70% of survivors and are particularly severe in children. Sequelae include parkinsonism, convulsive disorders, motor abnormalities, impaired intellect, and emotional disorders. The social prognosis of survivors is generally poor.

Diagnosis

Definitive diagnosis is similar to that described for SLE. In fatal cases, virus isolation and demonstration

of viral antigen by fluorescent antibody in brain tissue is feasible (175); isolations from blood or CSF are rare, but they are more likely during the first 4–5 days of illness. Serologic diagnosis depends on the demonstration of a fourfold or greater rise (or fall) in appropriately timed serum specimens. The HI, CF, and neutralization tests are applicable. Cross-reactions with heterologous viruses, particularly West Nile, may complicate the serodiagnosis in tropical areas of Asia. Serum IgM antibodies appear early, usually disappear by 3–6 months after onset, and are usually serologically specific (42,87). The IgM-capture ELISA is especially well suited for diagnosis by detection of locally synthesized antibody in the CSF (41,45), thus separating patients with virus infection of the CNS from those with other etiologies of encephalitis but serologic evidence for systemic (extraneural) JE infection. The kinetics of the systemic and local CNS antibody response have prognostic importance; patients who survive have earlier and more vigorous responses. IgM antibody synthesis persists in CSF for prolonged periods after recovery (89), possibly indicating persistent antigenic stimulation. Monoclonal epitope-blocking immunoassays may be used to identify JE-immunes among persons with cross-reactive flavivirus antibodies (43).

Treatment

There is no specific treatment. Good supportive care (as discussed for SLE) is essential. Vigilance should be maintained for severe hyperthermia, convulsions, and cerebral edema, and specific countermeasures should be applied. A technical guide to the clinical diagnosis and management of patients has been prepared by the World Health Organization (360).

Epidemiology

Morbidity and Mortality

JE virus is widely distributed in Asia, including Japan, China, Taiwan, Korea, Philippines, far-eastern USSR, all of Southeast Asia, and India. Fewer than 20 cases now occur annually in Japan. In China, large numbers of cases (>10,000) occur annually, but the annual incidence is declining. In contrast, epidemic activity in India, Nepal, and the northern part of Southeast Asia has increased since the early 1970s. Outbreaks in India between 1973 and 1983 have involved over 10,000 cases. In northern Thailand, annual outbreaks occur with attack rates of 10–20 per 100,000, an incidence similar to that of poliomyelitis in the United States before the advent of vaccines (160).

Case-fatality rates during epidemics range from 20% to 70%, but the high rates reflect poor medical care and recognition of only the most severe cases. Case-fatality rates in American servicemen varied between 2% and 11%. The elderly are at highest risk of fatal infection.

In endemic areas, children are primarily affected by the disease; attack rates in the 3- to 15-year age group are 5–10 times higher than in older individuals because of high background immunity in the older age groups. Epidemics in nonendemic regions have affected all age groups, but a bimodal age distribution (young children and the elderly) has appeared.

The ratio of inapparent to apparent infections is 200:1 to 300:1 (21). Among factors which influence this ratio are age, differing virus strain virulence, and cross-protective immunity to other flaviviruses, especially dengue (147).

In tropical areas there is an endemic pattern of infection, with occurrence of sporadic cases throughout the year. In temperate zones and in the northern part of the tropical zone, outbreaks have a marked seasonal incidence (July to September). Precipitation and temperature are important determinants of vector density and rate of virus transmission (217). An excess of cases has been noted in males in many outbreaks, presumably because of increased exposure in areas of rice cultivation.

The reasons for the absence of epidemics in tropical countries may be complex. In southern Thailand, intense JE virus transmission to pigs has been demonstrated in the absence of epidemic human disease (46). The RNA genome of virus isolates from this area differed significantly from northern Thai epidemic strains, suggesting that a difference in virulence may be responsible. Other factors to be considered include vector–host relationships, vector abundance, vector competence, and cross-protecting heterologous immunity to dengue.

Birds and pigs are effective viremic amplifying hosts (293). In temperate regions, the virus first appears in July in mosquitoes, followed within 1–3 weeks in pigs and birds, principally ardeid species (egrets, black-crowned night herons) and possibly also ducks. Human infections occur several weeks later. The main epidemic vectors are *Culex* species. *Culex tritaeniorhynchus* is the most important. It breeds in irrigated rice fields, shallow ditches, and pools; population densities peak during the hot summer months. Other species implicated in transmission include *Culex vishnui* (India), *Culex gelidus* and *Culex fuscocephalus* (Malaysia, Thailand), *Culex annulus* (Taiwan), and *Culex annulirostris* (Guam). Isolations have been made from many other species of *Culex*, *Anopheles*, and *Aedes*.

The overwinter maintenance of JE in temperate

areas has not been fully elucidated. Vertical transmission of JE virus by *Culex* and *Aedes* species has been demonstrated in the laboratory and by field studies (279). Other possible mechanisms include the following: survival in hibernating adult female *Culex tritaeniorhynchus*; maintenance in ticks, mites, or other alternate vectors; persistent infections in vertebrates; and reintroduction by migrating birds. JE virus and antibodies have been found in bats in Japan. Experimentally infected bats held in simulated hibernation develop persistent infections and, upon warming, have viremias sufficient to infect mosquitoes (184). JE virus and antibodies have been found in reptiles (300), but their role in the ecology of JE remains unknown. In tropical regions, year-round transmission of the virus between mosquitoes, birds, and pigs probably occurs (122).

Control

Vaccination

Vaccines have been used to prevent (a) encephalitis in horses and humans and (b) abortion and stillbirth in swine. Vaccination of horses with formalin-inactivated vaccines was the first successful application and afforded significant protection during an epizootic in Japan during 1948–1949. Since 1972, live attenuated vaccines have been licensed in Japan for use in pigs (362). Although immunization of pigs is a theoretical means of interrupting transmission and amplification of JE virus and thereby of preventing human infections, difficulties arise in practice. In many parts of Asia, pigs are only semidomesticated, and wide-scale immunization would be difficult. In areas such as Japan, where swine husbandry is highly developed, pigs are born after the summer epidemic period, have maternal antibody for 4 months, and are killed at 6–8 months of age, leaving a very narrow interval for vaccination.

Formalin-inactivated vaccines for use in humans are prepared from infected adult mouse brains or infected primary hamster kidney cell cultures in Japan and China, respectively. The mouse brain vaccine produced by the Research Foundation for Microbial Diseases (Biken), Osaka, Japan, is purified by protamine sulfate precipitation and ultracentrifugation and has been in wide use since the 1960s. A controlled trial of Biken vaccine in Thailand showed an efficacy of 91% (160). Mass vaccination campaigns have been carried out in Japan, Taiwan, and China, with children as the target population. Primary immunization requires two doses at a 7- to 14-day interval. Booster vaccinations are given during the first year after primary immunization and then at 3- to 4-year intervals (210). Side

reactions to the purified Japanese vaccines are relatively insignificant, and there have been no cases of postvaccinal encephalitis.

Because at least two antigenic variants of JE virus have been defined, a bivalent vaccine has been prepared incorporating Nakayama and Beijing-1 viruses (the latter being of JaGAr-O1 antigenic type). This vaccine has also proven efficacious in field trials (160). Attenuated live vaccines for human use have been developed and tested in China, with promising results.

Vector Control

Field trials of organophosphate larvicides and adulticides have proved effective against vectors of JE. Use of agricultural pesticides in rice-growing areas have also reduced populations of *Culex tritaeniorhynchus* (396). Integrated programs which include use of chemical larvicides, larvicidal fish, and biological larvicides (*Bacillus thuringiensis*), elimination of aquatic vegetation in irrigation canals, and spraying of residual insecticides in livestock pens have reduced the case incidence in China (161). Emergency epidemic control requires aerial ultra-low-volume spraying of organophosphate adulticides.

Molecular Approaches

RNA oligonucleotide fingerprints of virus isolates from human brains differed from isolates made from mosquitoes and pigs, and strains from epidemic areas differ from nonepidemic strains; these findings suggest the possibility that virus virulence may be an important epidemiologic determinant. The nucleotide sequences of two virulent JE virus strains have been reported (209,324), and studies of the Chinese live attenuated SA14-14-2 vaccine and its virulent parent are in progress. Given the expense and difficulty of manufacturing killed vaccines in mouse brain, there is a need for new vaccines produced by DNA technology.

Murray Valley Encephalitis

Epidemics of encephalitis ("Australian X disease") in southern Australia in 1917, 1918, 1922, and 1925 are now believed to have been due to Murray Valley encephalitis virus. In 1917 and 1918 an infectious agent was recovered by inoculation of monkeys (34), but the virus was not established or characterized. During an outbreak in 1951 the virus was isolated from human brain and was shown to be a flavivirus related to, but distinct from, JE virus (96). *Culex annulirostris* was suspected to be the vector in 1951; the virus was subsequently isolated from this mosquito in 1960 (84). Ep-

idemics occurred in southeastern Australia in 1956, 1971, and 1974. Small outbreaks also occurred in the Kimberley and Pilbara regions of western Australia in 1971, 1978, 1981, and 1984. The disease was recognized in New Guinea in 1956 (98).

Infectious Agent

Murray Valley encephalitis (MVE) virus is a member of the JE antigenic complex. Antigenic comparison of five MVE virus strains isolated in Australia and New Guinea by HI, kinetic HI, and plaque-reduction neutralization tests showed four strains to be similar and showed the fifth to have minor but reproducible antigenic differences. Strains from the 1974 epidemic were indistinguishable by standard serologic tests from isolates recovered during previous outbreaks (82).

MVE viruses analyzed by mapping restriction enzyme digests and by nucleotide sequencing showed a remarkable homogeneity of strains from widely separated areas of Australia over a 23-year period (192). This finding suggests continuing gene flow, possibly by widely ranging water bird hosts. Strains from New Guinea differ, suggesting that this area represents a separate focus. Monoclonal antibodies have been developed to define antigenic strain variation, but they have not yet been used. The partial nucleotide sequence of MVE has been reported (70).

Many vertebrate and mosquito cell culture systems propagate the virus. Plaque assays can be performed in primary chick embryo and continuous lines of pig kidney, monkey kidney, and hamster kidney cells (327,350). Immune enhancement of virus growth has been demonstrated in primary chick embryo fibroblast cultures mediated by a subpopulation of macrophages having Fc receptors (176). The host range of MVE virus has been reviewed by French (97). Infant mice are highly susceptible by all routes of inoculation. Mice develop clinical resistance to peripheral (but not intracerebral) inoculation between 17 and 28 days of age. Hamsters 6–10 weeks old are susceptible to lethal infection by all routes. Monkeys, horses, sheep, and some birds develop encephalitis after intracerebral inoculation. Pigeons and chickens infected subcutaneously develop viremic infections without clinical illness. Rabbits and guinea pigs have subclinical infections after intracerebral and peripheral inoculations. Chicken embryos are highly susceptible and have been used for primary isolation from human cases (96).

MVE virus is suspected to cause neurologic disease in horses, but field and experimental studies have failed to conclusively incriminate the virus (203). During epidemics of MVE, there are reports of dogs dying; dogs are susceptible to infection as shown by a high

antibody prevalence, but no evidence has been obtained for a pathogenic role of MVE virus.

Pathogenesis and Pathology

Relatively little is known of the experimental pathogenesis of MVE in laboratory animals. Pathological findings in human cases of fatal encephalitis are similar to those in JE (273).

Clinical Features

The disease begins with a 2- to 5-day prodrome characterized by fever, headache, myalgia, generalized malaise, anorexia, and nausea followed by the appearance of nuchal rigidity and neurologic signs. In infants the disease progresses rapidly, and patients are frequently comatose when first brought to medical attention. Bennett (22) divided patients into three groups on the basis of severity of illness: (a) mild cases with altered level of consciousness and variable neurological abnormalities, but without coma or respiratory depression, accompanied by stabilization of neurologic changes within 5–10 days; (b) severe cases with coma, paresis, and paralysis, including respiratory and pharyngeal impairment requiring respiratory assistance; and (c) fatal cases with spastic quadriplegia and progressive CNS damage or severe disease with superimposed bacterial infection. Neurologic sequelae occur in up to 40% of the mild cases and in all of the severe cases that recover. Deficits include paraplegia, impaired gait and motor coordination, and intellectual dysfunctions.

Attempts to associate MVE virus with mild febrile illnesses without neurological signs have failed (82).

Diagnosis

Specific diagnosis depends on isolation from brain tissues of fatal cases or serologic tests. Virus isolation from blood or CSF has not been successful. Isolations from brain have been made in chick embryos and suckling mice. For serodiagnosis, the HI, CF, and neutralization tests are useful. Cross-reactions with dengue and with Kunjin virus may confuse interpretation. Some patients with encephalitis (presumably MVE) have shown rising antibody titers to Kunjin (83), possibly on the basis of a previous Kunjin infection and the "original antigenic sin" phenomenon. IgM antibodies appear to be quite specific and useful for early diagnosis (355).

Treatment

Treatment is as described for St. Louis and Japanese encephalitis.

Epidemiology

Epidemics have occurred principally in the Murray Valley region of New South Wales and Victoria, involving up to approximately 50 human cases (203). The most notable recent outbreak, in 1974, was unusual in its geographical extent, with patients also infected in east central Queensland, Northern Territory, northern and southeastern South Australia, and the Ord River Basin of Western Australia. Sporadic cases were reported in New Guinea.

Outbreaks in Australia occur during the summer (January to May) and appear to follow periods of abnormally high rainfall for two consecutive years. Water impoundment and irrigation schemes were responsible for the emergence of MVE in Western Australia in the 1970s. A mathematical model of MVE has provided hypotheses regarding amplification and dissemination of the virus in southern Australia (173).

Population-based incidence data on MVE are not available. Prior to 1974, children were predominantly affected. In the 1974 epidemic, however, 35% of the patients were under 10 years old, and a similar proportion were over 50 years (83). A serologic survey conducted after the 1951 epidemic in various localities in Victoria showed 4.5–36% positive CF tests; the rate in children was about one-half that in adults (8). A statistically valid HI antibody survey conducted 8 months after the 1974 epidemic showed 4.5% positive overall; unexpectedly, no antibodies were found in persons 5–24 years of age (87). Very high MVE seroprevalence (over 50%) has been found in the Kimberley area of Western Australia and in the Murray–Darling River basin of New South Wales.

The principal vector is *Culex annulirostris*, a transient pool breeder. The virus has also been isolated from *Aedes normanensis*, *Culex tritaeniorhynchus*, and *Culex pipiens*. *Culex pipiens*, once considered to be a potential vector on epidemiological grounds, has proved to be poorly susceptible or refractory to oral infection in experimental studies (172). Experimental studies show *Culex annulirostris* to be a highly efficient vector (171).

Large water birds (such as herons, egrets, and pelicans) appear to be the most important viremic hosts. However, mammals (including rabbits and kangaroos) may contribute significantly to the transmission cycle.

The overwintering mechanism and origin of intermittent epidemics are unknown (for a review, see ref. 203). Even in tropical areas of Australia, year-round transmission of MVE virus has not been demonstrated. Vertical transmission has been shown experimentally in *Aedes aegypti*.

Prevention and Control

No vaccine is available. In areas prone to recurrent epidemics (e.g., the Murray River basin), reduction of

Culex annulirostris breeding by use of larvicides is practiced. Use of rainfall data and surveillance of virus activity in mosquitoes and sentinel fowl to predict risk of epidemics allows targeted mosquito control efforts.

Tick-Borne Encephalitis

Tick-borne encephalitis was clinically described in the Far Eastern Soviet Union in 1934. In 1937, Silber et al. isolated the causative virus from human brain and demonstrated tick transmission (310). The disease was first recognized in eastern Europe (Czechoslovakia) during an epidemic in 1948, and a virus isolated from a patient was shown to be similar to the Far Eastern virus. Transmission by ingestion of raw goat milk was demonstrated in both Russia and Europe. The disease has been recognized in all countries of eastern Europe and Scandinavia, as well as in France and Switzerland.

Infectious Agent

The tick-borne encephalitis (TBE) virus complex consists of 14 antigenically closely related viruses, eight of which cause human disease (Table 1). Russian spring–summer encephalitis (RSSE) and CEE are very closely related antigenically and have been considered subtypes of the same virus. They are separable on the basis of antibody-absorption HI, kinetic HI and CF tests, and agar gel diffusion (61,118). This antigenic distinction has been confirmed at the molecular level (152). The two subtypes are distinguished on the basis of peptide maps of both the E and the largest non-structural protein (NS5). As discussed below, the two subtypes also differ in their tick vectors and clinical expression. Remarkable stability of the CEE genome and antigenic determinants has been demonstrated among strains from different areas of Europe (129).

The morphology, chemistry, and antigenic composition of TBE complex viruses are similar to those of other flaviviruses (151,154,303). TBE complex viruses differ from the members of the genus, however, in their resistance to acidic pH. This feature is responsible for the occurrence of oral infection via milk, since the virus resists inactivation by gastric acid (253).

TBE complex viruses grow in a variety of cell cultures, including pig, bovine, and chick embryo, HeLa, Detroit 6, human amnion, Hep 2, Vero, and primary reptilian and amphibian cells (261). Cytopathic effect and plaquing are variable.

Chicken embryos and a variety of animals are susceptible to infection. Infant and weanling mice develop fatal encephalitis by all routes of inoculation. Rats, guinea pigs, sheep, monkeys, and swine succumb to encephalitis after intracerebral inoculation. Hamsters are less susceptible to intracerebral and peripheral

challenge than mice (304). Experimental inoculation of wild vertebrate species (including rodents, insectivores, foxes, birds, hares, and bats) results in viremia and antibody formation (118). Cows, goats, and sheep experimentally infected by inoculation or tick bite develop viremia and secrete virus in their milk. Mice can also be infected by the oral route, with subsequent shedding of virus in feces and milk (254).

The Far Eastern virus type (RSSE) is more virulent for sheep and monkeys inoculated intracerebrally than the Western (CEE) virus (364). However, no distinct difference has been noted in pathogenicity for mice or cell cultures (18). Variation has been found between wild virus strains in their ability to produce viremia in *Clethrionomys glareolus* voles (59).

Pathogenesis and Pathology

The pathogenesis in mice was reviewed by Albrecht (5) and does not differ significantly from the general scheme presented earlier. In monkeys the anterior horn cells of the spinal cord and cerebellar cortex appear to be selectively more vulnerable to tick-borne viruses than other neuronal centers (237). An apparently unique feature of members of the TBE complex may be their propensity to cause persistent infections in experimental animals (and possibly also in humans). Mice infected with Kyasanur Forest virus may survive for months with paralysis, low titers of virus in the brain, and absence of detectable neutralizing antibodies (259). Hamsters infected with CEE virus develop late CNS lesions, including proliferation and hypertrophy of astrocytes (367), and have detectable viral antigen in tissues for up to 4 months. Louping-ill virus persisted in immunosuppressed guinea pigs for 50 days (366). Monkeys infected intranasally or intracerebrally with CEE virus developed a chronic encephalitis with degenerative spongiform lesions and astrocytic proliferation (367). The virus has been isolated from monkey tissues by cocultivation and explant procedures as long as 383 days after inoculation (256). Chronic progressive human encephalitis and seizure disorders (Kozhevnikov's epilepsy) have been associated with RSSE virus on serological grounds (245), and virus has been isolated from the CSF of a patient with amyotrophic lateral sclerosis (233).

The neuropathology of experimental infection in mice has been reviewed by Vince and Grcevic (340), and the pathology of infection in humans has been reviewed by Zilber and Soloviev (365). Findings are generally similar to those of other flaviviral encephalitides. Gross changes include swelling, congestion, and petechial hemorrhages. Histopathologic alterations include meningeal and perivascular inflammation, neuronal degeneration and necrosis, neuronophagia, and glial nodule formation involving cerebral and cerebel-

lar cortex, brain stem, basal ganglia, and spinal cord. The anterior horn cells of the cervical cord are especially vulnerable, explaining the predominance of lower motor neuron paralyzes of the upper extremities seen in many cases.

Clinical Features

The incubation period is 7–14 days. The disease in the Far East (RSSE) differs clinically from CEE. Onset of illness is more often gradual than acute, with a prodromal phase including fever, headache, anorexia, nausea, vomiting, and photophobia. These symptoms are followed by stiff neck, sensorial changes, visual disturbances, and variable neurological dysfunction, including paresis, paralysis, sensory loss, and convulsions. In fatal cases, death occurs within the first week after onset. The case-fatality rate is approximately 20% (117). The disease in children is more severe than in adults. Neurologic sequelae occur in 30–60% of survivors, especially residual flaccid paralyzes of the shoulder girdle and arms.

The Central European form is milder and has a typical diphasic course in approximately 50% of cases. The first phase is nonspecific and grippelike, lasting about 1 week and followed by a 1- to 3-day remission. The second phase begins abruptly; it may take the form of benign meningitis, or, in more severe cases, encephalitic signs appear, predominantly tremor, dizziness, altered sensorium, diplopia, and paresis (263). The case-fatality rate is 1–5%. Approximately 20% of survivors have objective neuropsychiatric sequelae, which tend to be minor in degree (264), and paralyzes are rare. CSF changes are similar to those in the other flavivirus encephalitides.

Diagnosis

Fewer than half the patients give a history of tick bite. Consumption of unpasteurized milk, seasonal incidence, and occurrence of other cases in the community may provide clues to the etiology. The Far Eastern disease may mimic poliomyelitis.

Definitive diagnosis depends on virus isolation or serology. The virus may be isolated from the blood during the first phase of illness and from brain tissue of patients dying early in the infection. Infant mice, embryonated eggs, and chick embryo cell cultures (with detection of virus by interference assay or immunofluorescence) have been used for virus isolation; the success rate in any series of patients is, however, less than 10%.

Serological diagnosis depends on the demonstration of significant changes in antibody titer between appropriately timed specimens. The HI, CF, single radial hemolysis, and neutralization tests have been used. CF

antibody responses are quite variable, and CF antibodies may not appear at all (183).

Diagnosis by estimation of IgM antibodies has replaced other assays (277). The test is valuable for rapid diagnosis and is applicable to both serum and CSF.

Epidemiology

TBE occurs in an endemic pattern over a wide area of Europe and the Soviet Union, corresponding to the distribution of ixodid tick vectors (Fig. 10). The annual incidence is several thousands of cases, with considerable variation from year to year. Incidence rates in Czechoslovakia ranged from 5 to 20 per 100,000 during the period 1945–1960 (27). The disease occurs in foci characterized by ecological habitats favorable for ticks. The intensity of transmission varies from year to year; increases in small-mammal populations (the principal hosts for immature ticks), with subsequent migration, are followed within 1–2 years by increased tick populations and a higher risk of human infections.

The virus is maintained in nature in a cycle involving ticks and wild vertebrate hosts. At least 10 species of rodents have been implicated as amplifying hosts (55,203). Insectivores—shrews, moles, hedgehogs—which have relatively stable populations (in contrast to rodents), are believed to be important reservoirs. Large mammals, such as goats, sheep, and cattle, serve as hosts for adult ixodid ticks, but viremia levels are probably too low to infect vectors (242). The virus is excreted in the milk, however, and human infection may result from consumption of unpasteurized goat's or sheep milk or cheese. Vertebrate hosts may also be involved in overwintering. Prolonged viremic infections have been demonstrated in hibernating dormice and hedgehogs, bats, and ducks.

Ixodes ricinus and *Ixodes persulcatus* are responsible for transmission in Europe and the Soviet Union, respectively (Fig. 10). Other tick species, of the genera *Dermacentor* and *Haemaphysalis*, have also been implicated in transmission, especially in areas that do not

support *Ixodes* ticks. Pre-imago ticks acquire infection by feeding on viremic small mammals and pass the virus transtadially to the adult stage. Transtadial and transovarial virus transmission has been demonstrated in *Ixodes*, *Dermacentor*, and *Haemaphysalis*. Considerable losses of virus occur with tick moulting from stage to stage. Approximately 1% of the progeny of an infected female *Ixodes* acquire the virus. Although ticks may serve as a natural reservoir and overwintering mechanism, horizontal transmission between vectors and vertebrates is required for endemicity. A mathematical model has been described which predicts age-specific infection rates and infection risk in hyperendemic foci (110).

Transmission to humans occurs primarily in adults over 20 years old who live, work, or vacation in rural, natural foci; persons at highest risk are farmers, shepherds, forestry workers, campers, and so forth. The disease occurs in two peaks (May to June and September to October), coinciding with the activity of adult *Ixodes* ticks. Small outbreaks, involving all age groups and often in family groups, result from consumption of raw sheep or goat's milk or cheese (119). Laboratory infections with TBE virus are not uncommon (294). An imported case of TBE occurred in a child in Ohio (68).

The molecular epidemiology of TBE has been studied by Heinz and Kunz (152). RSSE and CEE viruses are distinguishable by peptide mapping of the E and NS5 proteins. However, strains from Europe (including Scandinavia) isolated over a 26-year period are quite homogeneous, as determined by monoclonal antibody analyses, peptide mapping, and competitive RIAs. This high degree of genetic homogeneity is distinct from mosquito-borne flaviviruses that have been studied (dengue and SLE).

Prevention and Control

Prior to World War II, formalin-inactivated mouse brain vaccines were used in the USSR. Subsequently,

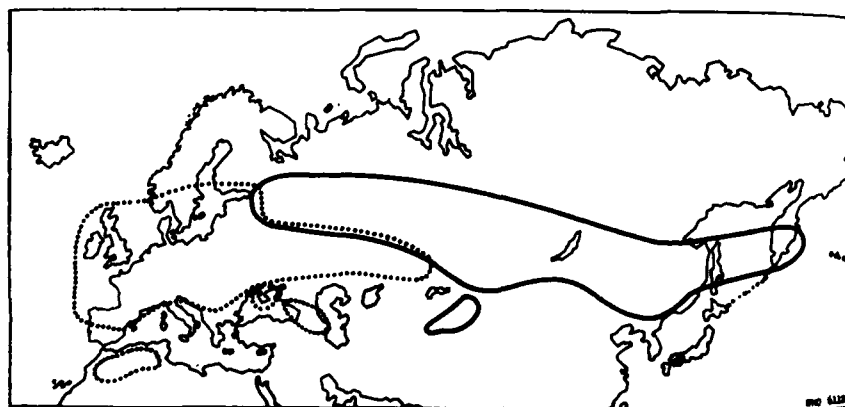


FIG. 10. Distribution of *Ixodes ricinus* (vector of Central European encephalitis and louping-ill viruses, dotted line) and *Ixodes persulcatus* (vector of Russian spring-summer encephalitis, solid line). (From ref. 27 with permission.)

inactivated vaccines have been produced in embryonated eggs or chick embryo cell cultures (188). Czechoslovakian and American workers have studied attenuated clones of Langat virus as a potential immunizing agent which cross-protects against TBE (208). The most advanced approach to vaccination, however, is that of a cooperative Austrian-British project, using a chick embryo cell culture-grown virus partially purified by hydroxylapatite chromatography or continuous-flow zonal ultracentrifugation and inactivated with formalin (182). The vaccine contains $Al(OH)_3$ as an adjuvant, produces serological conversions in over 95% of recipients, and has provided 99% protection in field efficacy trials. Vaccination is recommended for persons inhabiting or moving into natural disease foci or persons working under high-risk conditions (foresters, woodcutters, laboratory workers).

The following are important preventive measures: avoidance of tick bite by use of repellents and protective clothing; health education; and pasteurization or boiling of raw milk. In the past, widespread use of pesticides, including aerial applications of DDT, has been undertaken in some areas in attempts to interrupt transmission.

Other Viruses Causing Encephalitis

Rocio Virus

Rocio virus was first isolated from fatal human cases in 1975 during an explosive outbreak of encephalitis on the south coast of Sao Paulo State, Brazil (194,195). The virus is not placed in any of the flavivirus antigenic complexes, but it cross-reacts most closely with members of the JE subgroup. It is pathogenic for infant mice and hamsters and adult mice by all routes of inoculation. Adult hamsters are susceptible to intracerebral inoculation. Guinea pigs survive peripheral inoculation, and newborn chicks develop viremia but rarely show signs of illness. The virus grows to high titer and forms plaques in Vero, BHK, and porcine kidney cell cultures. The pathologic changes in humans are typical of other flavivirus infections; the most severely affected structures in seven patients studied were thalamus, dentate nucleus of the cerebellum, hypothalamic nuclei, and substantia nigra (284). In experimentally infected suckling hamsters, Rocio virus produced severe necrosis of myocardium and pancreas (144).

The clinical features in human cases are similar to those of St. Louis and Japanese encephalitis (329). The case-fatality rate in hospitalized patients was 4%. Sequelae, including persistent cerebellar, motor, and neuropsychiatric signs, were noted in 20% of survivors. Diagnosis is by virus isolation from brain tissues of fatal cases or by serology.

Epidemics in coastal Sao Paulo State in 1975, 1976, and 1977 resulted in 821 reported cases, with attack rates of up to 38 per 1,000 inhabitants (163,195). Since 1978, sporadic cases have occurred, and the virus is presumed to be endemic. The highest disease incidence is in young adult males engaged in outdoor work in impoverished agricultural areas. There was no associated disease in domesticated animals. Wild birds are presumed to be viremic hosts in the transmission cycle. A single virus isolation was made from *Psorophora ferox* mosquitoes. *Aedes serratus* and *Aedes scapularis* were implicated on epidemiologic grounds (95). Both *Psorophora ferox* and *Aedes scapularis* were found to experimentally transmit Rocio virus, whereas *Aedes serratus* did not (216). A mouse brain vaccine has been evaluated but was shown to lack potency (196).

Louping-ill

Louping-ill was recognized as a neurological disease of sheep in Scotland during the late nineteenth century. Isolated in 1929 (257), the virus is a member of the TBE virus complex. The virus kills suckling mice by all routes of inoculation; weanling mice, hamsters, and guinea pigs develop fatal encephalitis after intracerebral inoculation. Many cell cultures propagate the virus; plaque assays may be performed in porcine kidney, Vero, and LLC-MK2 cells. Experimentally infected sheep develop prolonged viremia followed by ataxia, paralysis, and tremors (267). Pathologic changes include diffuse meningoencephalitis, with severe chromatolysis and destruction of Purkinje cells, reactive gliosis, and astrogliosis. Virus strain differences have been noted in virulence for sheep. Concurrent *Erlichia* infection (tick-borne fever) and external stress (shipping, cold, etc.) enhance the disease. Experimentally infected red grouse also develop fatal infection and viremias sufficient to infect tick vectors (266). Louping-ill is predominantly a disease of sheep, which develop a biphasic illness; the first phase is characterized by fever and weakness, followed by a neurologic illness with prominent cerebellar ataxia, hyperexcitability, and progressive paralysis. Sporadic cases in cattle and epornitic disease in wild red grouse also occur (268). The human disease was first described in a person with a laboratory infection (272). Approximately 35 human cases have been reported, of which 26 resulted from laboratory exposure (306). Of the natural infections, some followed tick bites whereas others followed direct exposure to sick sheep.

The human disease is biphasic and resembles CEE. The incubation period is 4–7 days. The first phase is influenza-like, lasting 2–11 days, followed by a remission of 5–6 days and then the reappearance of fever and a meningoencephalitic syndrome that last 4–10

days. There is leukopenia during the first phase and leukocytosis during the encephalitic phase. No deaths have been reported. The clinical features are reviewed by Smith and Varma (306) and Webb et al. (346). In one laboratory-acquired case, a hemorrhagic diathesis developed, and the disease closely resembled Kyasanur Forest disease. Treatment is symptomatic. Diagnosis is by virus isolation from blood during the first phase of illness, from CSF during the early encephalitic phase, or by serologic tests (HI, CF, neutralization). Antibodies are produced locally in the CNS. The serum/CSF antibody ratio has been employed in human diagnosis (346).

Louping-ill virus is distributed in Scotland, northern England, Wales, and Ireland. The transmission cycle involves *Ixodes ricinus* ticks and both sheep and grouse. Control of the disease in sheep is by vaccination, dipping with residual acaricides, and environmental control of ticks. The vaccine now in general use is grown in sheep kidney cell cultures, is formalin-inactivated, and is concentrated by methanol precipitation (39). A killed vaccine for use in laboratory and abattoir workers was once used on an experimental basis (90).

Powassan

Powassan virus was first isolated in 1958 from a fatal case of encephalitis in Ontario (214). It is a member of the TBE complex but is more distantly related than other viruses in the complex (48). The virus is pathogenic for infant and weanling mice by the intracerebral and intraperitoneal routes; hamsters and rabbits develop subclinical infections. Experimental encephalitis has been demonstrated in rhesus macaques. Continuous cell lines of monkey kidney origin are useful for virus assay by CPE and plaque formation. Pathologic changes in the brains of mice, monkeys, and humans are typical of other flavivirus infections (214). Powassan infection is characterized by a variable period of fever and nonspecific symptoms, followed by neurologic signs of meningeal irritation and encephalitis which are often severe. Sequelae are frequent, and in at least one case they resembled RSSE with shoulder-girdle involvement (65). In another case, the disease closely mimicked herpes encephalitis with temporal lobe involvement.

Powassan virus has a wide distribution in the eastern and western United States and Canada; human disease has occurred only in Ontario and the eastern United States. The virus has been isolated from ticks (*Haemaphysalis neumannii*) and mosquitoes in the Soviet Union, where it has also been reported to cause human infection and encephalitis (186). Approximately twenty cases, mostly in children, have been reported

in the United States and Canada, with one death. Treatment is symptomatic; diagnosis is by serology or virus isolation from brain tissues of fatal cases. In North America the virus is transmitted in a cycle involving small mammals (principally squirrels and ground hogs) and *Ixodes* ticks, including (a) *Ixodes marxi* and *Ixodes cookei* in the east and (b) *Ixodes spinipalpus* in the western states (11,12). The virus has also been isolated from *Dermacentor andersoni* in South Dakota. Serologic surveys and virus isolations have shown infections in wild mammals, including rodents, hares, dogs, skunks, and mustelids. In an experimentally infected lactating goat, virus secreted in the milk resulted in infection of the offspring; antibody has been demonstrated in naturally infected goats in New York State, indicating the possibility of milk-borne transmission to humans (358). Human infections are rare, however; antibody surveys have generally shown prevalence rates of less than 1%. No special control measures are indicated.

Negishi

Negishi virus was isolated from the CSF of a fatal human case in Tokyo in 1948 (2). The virus is a member of the TBE virus complex (61). Monoclonal antibody analysis showed the virus to be most closely related to Langat virus in the TBE complex and also showed an unexpected relationship to JE virus (149). Infant and weaned mice are highly susceptible to peripheral and intracerebral infection. A second fatal case occurred in 1948, and in 1950 a laboratory infection was reported with fever but no neurologic signs (246). Unpublished data indicated the occurrence of human cases of Negishi encephalitis in China.

FLAVIVIRUSES ASSOCIATED PRIMARILY WITH FEVER-ARTHRALGIA-RASH

Dengue Fever

The first epidemic of a disease resembling dengue was described in Philadelphia in 1780 by Benjamin Rush. Epidemics were common during the eighteenth and nineteenth centuries in North America, the Caribbean, Asia, and Australia (52,301). Transmission by *Aedes aegypti*, first described by Bancroft in 1906, was later proved by Siler et al. (301) and Simmons et al. (302). Ashburn and Craig found a filterable, infectious agent in human blood in 1906; in 1926 and 1931, respectively, Siler et al. (301) and Simmons et al. (302) transmitted the virus to human volunteers and established the incubation period in mosquitoes. The virus was isolated in mice by Sabin and Schlesinger in 1944 (290), and the existence of more than one serotype was

established by cross-protection studies in human volunteers (289).

Dengue is a world-wide public health problem; epidemics involving thousands of persons recur in areas of tropical Asia, Africa, and America where *Aedes aegypti* is present. A severe, occasionally fatal form of the disease was recognized in Asia in 1954 (see section entitled "Dengue Hemorrhagic Fever," below).

Infectious Agent

Four serologic types (types 1–4) are recognized on the basis of plaque reduction neutralization tests (287) and constitute a distinct antigenic complex (73). Dengue types 1 and 3 form a subcomplex defined by monoclonal and polyclonal antibodies (155). Some antigenic heterogeneity is apparent between strains of each type by conventional neutralization tests (286). However, monoclonal antibodies have also shown unexpected relationships at the subcomplex level [e.g., between dengue 2 and 4 and between dengue and viruses of the JE and TBE complexes (reviewed in ref. 143)]. At a more detailed level, antigen signature analysis of dengue 2 strains by monoclonal radioimmunoassay (RIA) has revealed antigenic variation that correlates with genomic variation demonstrated by RNA oligonucleotide fingerprinting (230). The latter technique has now defined at least 14 geographic variants of dengue 2 virus (see section entitled "Epidemiology," below).

Nucleotide sequences of dengue types 1, 2, and 4 have been reported (77,132,205). Within the dengue complex, amino acid sequence positional homology of 63–68% was found, compared to 44–51% between dengue, yellow fever, and West Nile viruses. Genetic variants of dengue 2 show more than 90% similarity. A relationship reported between dengue 2 virus and Edge Hill virus by RNA–DNA hybridization (28) has not been investigated at the sequence level.

Dengue viruses grow in a variety of primary and continuous cell cultures; high yields and demonstration of CPE are difficult to obtain in many systems without adaptation and passage. Cells of human (BSC-1), monkey (LLC-MK2, Vero, primary monkey kidney), hamster (BHK-21), and mosquito origin are most susceptible. Yields of up to 7 dex, CPEs, and plaque formation are obtained under appropriate conditions.

Dengue viruses replicate in the brains of suckling mice and hamsters inoculated intracerebrally. However, unadapted virus strains usually produce subclinical infections or only scattered illness with paralysis and death. Guinea pigs, rabbits, cotton rats, adult hamsters, chickens, and lizards are not susceptible to infection (170,295). Embryonated eggs replicate some strains only after repeated passage. Adult mice

inoculated with highly adapted dengue types 1 and 2 viruses become infected with or without overt encephalitis (30). Old World and New World monkeys and apes develop subclinical infection and viremia (278,302,354).

Dengue viruses replicate to high titer in *Aedes* spp. and *Toxorhynchites* spp. mosquitoes inoculated intrathoracically or intracerebrally (283).

Pathogenesis and Pathology

Neuroadapted dengue virus produces typical encephalitic lesions, predominantly in the rhinencephalus of infant, weanling, and adult mice. Viral antigen is detectable by immunofluorescence in reticulo-endothelial cells of liver, lymph nodes, and spleens of intraperitoneally infected mice (30). Athymic nude mice peripherally infected with adapted dengue virus develop fatal encephalitis and viral antigen in neurons, skeletal muscle, myocardium, and Kupffer cells.

In experimentally infected nonhuman primates, the role of mononuclear phagocytes as principal sites of dengue viral replication has been established by tissue titration and immunofluorescent staining of cells in skin, spleen, lymph nodes, liver, lung, and thymus; *in vitro* infection of monocytes support these findings. Mild inflammatory lesions are found in the brains of monkeys inoculated by the intracranial route.

Classic dengue fever produces self-limited infection in humans. Biopsies of skin lesions have shown swelling of endothelial cells of small vessels, perivascular edema, and infiltration of mononuclear cells.

Dengue viruses multiply in the midgut epithelium, brain, fat body, and salivary glands of mosquitoes (283). No detectable pathologic changes result from infection, and mosquitoes remain infectious for life. Dengue virus replicates in the female mosquito genital tract and may enter the ovum at the time of oviposition, thereby infecting a portion of her progeny (281). Sexual transmission also occurs from male *Aedes* with inherited infections to susceptible females, which may subsequently pass the virus to their progeny; sexual transmission from female to male has not been shown (282).

Clinical Features

The clinical manifestations of dengue fever were described by Siler et al. (301), Simmons et al. (302), and Sabin (289). In the typical case, the disease begins abruptly, after a 2- to 7-day incubation period, with high fever, headache, retrobulbar pain, and lumbosacral aching pain. Fever may be sustained for up to 6–7 days or may have a biphasic (saddle-back) course. Initial symptoms are followed by: generalized myalgia or bone pain that increases in severity; anorexia; nau-

sea; vomiting; weakness; and prostration. The pulse rate may be slow in relation to the fever. Respiratory symptoms (cough, sore throat, and rhinitis) are not uncommon, especially in children. A transient, generalized macular or mottled rash may appear on the first or second day. Coincident with defervescence (day 3–5) or shortly thereafter, a secondary rash, maculopapular or morbilliform in nature and nonirritating, appears first on the trunk and then spreads centripetally to the face and limbs but spares the soles and palms. The rash may desquamate. Fever may rise again, creating the second phase of the saddle-back course. Generalized lymphadenopathy, cutaneous hyperesthesia, and altered taste sensation may accompany this step of the disease. The peripheral WBC count is depressed with an absolute granulocytopenia, and the platelet count may fall to $<100,000/\text{mm}^3$. Hemorrhagic phenomena are noted in a few cases and include petechiae, epistaxis, intestinal bleeding, menorrhagia, and a positive tourniquet test.

Toxorhynchites mosquitoes are sensitive hosts for dengue virus isolation by intrathoracic inoculation; virus can be identified by immunofluorescence staining or CF tests on mosquito tissues within 10–14 days after inoculation (179). *Toxorhynchites amboinensis* (TRA-284), *Aedes albopictus* (C6/36), and *Aedes pseudocutellaris* (AP-61) cell lines are now widely used for primary dengue virus isolation (181). The TRA-284 line adapted to serum-free medium provides the most sensitive assay. Syncytial CPE may be present, but it is an unreliable marker of infection; moreover, cultures must be examined by immunofluorescence (IF) to detect virus. Monoclonal antibodies are used for type-specific identification by IF. These techniques allow isolation and identification in as short a period as 2 days, depending upon titer of virus in the test sample.

Direct detection of dengue viral antigen in human serum has been reported by use of countercurrent immunoelectrophoresis and by monoclonal RIA (231).

Serologic diagnosis depends on the demonstration of a fourfold or greater rise (or fall) in antibodies by the HI, CF, or neutralization test. Radio- or enzyme-linked and thin-layer immunoassays are also applicable. It is frequently difficult to establish the specific infecting serotype because of cross-reactions, especially in individuals with preexisting heterologous immunity. In the case of sequential dengue infections, the antibody response to the initial infecting virus type may exceed that to the current infecting type ("original antigenic sin") (138). Myocarditis and various neurologic disorders have been associated with dengue fever. Neurologic manifestations include encephalopathy and peripheral mononeuropathy or polyneuritis (125). Central neurologic disorders appear to be more common in dengue hemorrhagic fever than in

classic dengue. Reye's syndrome has also been reported to follow dengue infection.

Convalescence may be prolonged, with generalized weakness, depression, bradycardia, and ventricular extrasystoles. Persistent arthritic symptoms are not a feature of dengue and suggest other viral etiologies, including alphavirus (Ross River or chikungunya) infection.

Diagnosis

Exposure by residence or travel in dengue-endemic areas and knowledge about the occurrence of other cases in the community are important clues to the diagnosis. Other infections that clinically may be confused with dengue include influenza, rubella, rubeola, malaria, scrub typhus, leptospirosis, and a variety of other arboviral infections. Rash is a helpful differential sign, but it may be difficult to discern in dark-skinned persons. Epidemic arboviral infections that resemble dengue fever and may be accompanied by rash include chikungunya, o'nyong nyong, West Nile, Sindbis, Mayaro, and Ross River virus diseases.

Specific diagnosis depends on virus isolation or serologic tests. Virus may be recovered from the blood during the early febrile phase of the illness. Viremia titers in dengue 1, 2, and 3 infections range from barely detectable to 8 dex for 3–5 days; titers in patients infected with dengue 4 are approximately 100-fold lower (126). The plaque-reduction neutralization (N) test is more specific than other tests. Epitope-blocking immunoassays employing monoclonal antibodies provide diagnostic specificity similar to the N test (43). The IgM-capture ELISA has replaced other serological techniques in some laboratories; IgM antibodies indicate recent dengue infection but do not generally provide a type-specific diagnosis in cases of dengue or flavivirus superinfection.

Treatment

Treatment is supportive and includes bed rest, antipyretics, and analgesics. In case of dehydration, fluid and electrolyte replacement are used in addition.

Epidemiology

Dengue occurs principally in tropical areas of Asia, Oceania, Africa, Australia, and the Americas. Temperate areas within the range of *Aedes aegypti* are susceptible to summertime introduction and spread of the virus. In areas having year-round vector activity and large human populations, one or more dengue virus types may be maintained endemically. Elsewhere, es-

pecially in small, insular populations, epidemics result from the introduction of a new type.

Protection against homotypic reinfection is complete and probably lifelong, but cross-protection between dengue types lasts less than 12 weeks (289). Experimental infection in nonhuman primates has also shown incomplete cross-protection between pairs of dengue viruses, and sequential infection with three or four dengue types is required to achieve complete protection. The background of immunity of human population groups determines the incidence and age distribution of infections.

Morbidity estimates in various epidemics are reviewed by Ehrenkranz et al. (91) and Brès (35). Some outbreaks have involved 1 million or more cases, with attack rates of 50–90%. In 1986 in the Americas, approximately 88,000 cases were reported, whereas the true morbidity was estimated to have exceeded 2 million. The world-wide incidence of dengue has increased dramatically in the period following World War II, due to expanding urban human populations and a coincident increase in *Aedes aegypti* density, as well as the advent of air travel and rapid movement of viremic persons. Large epidemics in recent years have followed introduction of dengue virus from afar (e.g., outbreaks of dengue 2 and 3 in East Africa and of dengue 2 and 4 in the Americas in the 1980s).

In hyperendemic areas of Southeast Asia, over 50% of children experience infection with one or more dengue serotypes by age 7. In tropical areas, epidemics tend to occur during the monsoon or rainy season. Although increased rainfall results in expansion of vector mosquito breeding, human disease incidence does not correlate closely with vector population density. Other factors (in particular, increased temperature, which shortens the extrinsic incubation time of dengue virus in the vector) appear to be more important (344).

Dengue virus is transmitted in a cycle involving humans and mosquitoes. *Aedes aegypti* is the most important vector, but other species, including *Aedes albopictus*, *Aedes polynesiensis*, and *Aedes scutellaris*, also play a role in rural and insular areas of Asia and the Pacific. *Aedes aegypti* breeds in artificial containers with clean water in and around human habitations and bites principally during the daytime. The mosquito is furtive in habit, and feedings are frequently interrupted, resulting in multiple human infections. In addition to biologic transmission, mechanical spread by *Aedes aegypti* and other mosquitoes may occur (302).

Geographic variation exists between strains of *Aedes aegypti* and *Aedes albopictus* in terms of their vector efficiency (susceptibility to oral infection), but the epidemiologic importance of this variation has not been clearly established (127).

Zoonotic cycles of dengue virus transmission involving monkeys and forest *Aedes* spp. have been doc-

umented in Malaysia (285) and West Africa (67,274). The vector in Malaysia is *Aedes niveus*; the species implicated in West Africa are *Aedes furcifer*, *Aedes taylori*, *Aedes luteocephalus*, *Aedes opok*, and *Aedes africanus*. The possibility of a forest cycle in South America deserves study.

The maintenance of dengue viruses between epidemics has not been clearly defined, especially in rural areas with relatively sparse human populations. An alternative mechanism to year-round horizontal human-mosquito-human transmission, such as vertical transmission in *Aedes* vectors, must be considered. Similar considerations apply to the maintenance of the sylvatic cycle over the dry season. Experimental studies have documented vertical transmission of dengue virus in *Aedes*, and the virus has been isolated from field-collected larvae of *Aedes aegypti* and males of *Aedes furcifer-taylori* in West Africa (for reviews, see refs. 123 and 124).

Molecular Approaches

RNA oligonucleotide fingerprinting, RNA-DNA hybridization, and antigenic analysis have been useful tools in determining the origin and spread of dengue epidemics (174,230,331). By these techniques, each serotype may be classified into a number of geographic variants or topotypes (as many as 14 dengue 2 topotypes are currently recognized). Within a single geographic area, genetic changes in the virus population may be found over time (342), with the appearance of new genotypes by mutation and selection as well as by introduction from afar. For example, a new (Jamaican) genotype of dengue 2 appeared in the Caribbean in 1981 and now coexists in multiple areas with the Puerto Rican topotype present since at least 1969. The similarity between the Jamaican topotype and a strain from West Africa suggested a possible source of introduction. Similarity between the Puerto Rican topotype and strains isolated in the South Pacific between 1971 and 1976 also suggested routes of spread of dengue 2 epidemics.

Prevention and Control

An attenuated suckling mouse brain dengue type 1 vaccine provided significant protection to a small number of vaccinees in Puerto Rico in 1963 (356) but is not available for use.

More recently, an attenuated, low-plaque, temperature-sensitive dengue 2 vaccine (S-1 clone) produced in cell culture was found to induce neutralizing antibodies only in persons with prior yellow fever immunity (15). Another candidate dengue 2 vaccine (16681-PDK53) with similar virologic characteristics

has been developed; this vaccine does not produce symptoms and is immunogenic in flavivirus nonimmunes (25). Live attenuated vaccines to other dengue serotypes are in various stages of development.

Various strategies are also being explored toward the development of genetically engineered vaccines. A dengue 4 baculovirus recombinant expressed both E and NS1 proteins in insect cell culture, and the protein products induced protective immunity in mice (363). Purified native NS1 protein has also been shown to induce protective immunity in mice (298). This novel approach to immunization, which does not involve virion antigens and thus would not induce enhancing antibodies, is of great theoretical importance, given the presumed pathogenesis of dengue hemorrhagic fever.

Vaccine development has been linked to the needs of high-risk military groups but may eventually prove useful for general populations, especially for protection against epidemic dengue hemorrhagic fever. Because of the lack of cross-protection between serotypes, multivalent immunization will be required.

Prevention of epidemics relies principally on reduction or eradication of *Aedes aegypti* by breeding site elimination, use of larvicides, and perifocal spraying of insecticides. Eradication achieved 20 years ago in many countries of Latin America has now been reversed by repeated reinfestations, economic development with attendant expansion of *Aedes aegypti* breeding, reduction in program support, and insecticide resistance, but the efficacy of this approach is in question.

For the emergency control of epidemics, it is necessary to interrupt transmission by killing infected adult female *Aedes aegypti*. Ultra-low-volume aerial or ground applications of organophosphate insecticides have been used (136,240).

West Nile Fever

Infectious Agent

West Nile virus is a member of the antigenic complex of flaviviruses which includes Murray Valley, St. Louis, and Japanese encephalitis viruses. The structures of the West Nile virus genome and glycoprotein spike have been partially analyzed (243). Kunjin virus (another agent in the complex) is more closely related to West Nile than to other members of the complex (64,350). A high degree of cross-protection was found in hamsters immunized with Japanese or St. Louis encephalitis viruses and challenged peripherally with West Nile (141).

There is a considerable body of research on antigenic variation of West Nile virus. Strains from Africa, Europe, the USSR, and the Middle East as far east as

Pakistan form one antigenic group distinct (by HI, kinetic HI, CF, and other techniques) from strains isolated in India and the Far East (103,260). These results have been interpreted to indicate frequent intercontinental exchange of West Nile virus strains by migrating birds. Other studies have shown considerable heterogeneity among strains isolated within a single region (23).

West Nile virus grows and produces CPEs or plaques in a wide variety of cell cultures (including primary chick and duck embryo), as well as in continuous lines of human, primate, swine, rodent, and amphibian origin. It multiplies in *Aedes aegypti* and *Drosophila* cells and produces CPE in *Aedes albopictus* cells.

Mice and hamsters of all ages are susceptible to lethal infection by the intracerebral route. Resistance to peripheral routes of inoculation develops with age; some virus strains are pathogenic for adult animals. Lethal oral infection of adult mice has been described. Hamsters transmit virus to their young via the milk. Rabbits, guinea pigs, and cotton rats develop antibodies without overt illness by all inoculation routes; rats succumb to intracerebral infection only (240). Almost all birds tested develop viremia, including wild species, chickens, and pigeons; encephalitis and death may occur but are rare (210,325). With one exception (namely, *Arvicanthus abyssinicus*), wild African rodents do not develop viremia.

Two isolated cases of naturally acquired West Nile encephalitis have been reported in horses (128); however, low-level viremia, antibody production, and absence of clinical illness are the rule. Bovine species do not develop viremia after experimental inoculation, but antibodies in cattle are prevalent. Rhesus monkeys develop encephalitis (occasionally fatal) after intracerebral or intranasal infection; cynomolgus monkeys develop fever without nervous system signs. The developing chick embryo is highly susceptible to the virus. Changes in hepatic regulatory enzymes and glycolytic and lipogenic pathways have been demonstrated in chick embryos (185).

Strain variation exists in the pathogenicity of West Nile virus for cell cultures and mice (338). Experimental infections in various arthropods have been reported (for a review, see ref. 147). *Culex univittatus*, the principal vector in Africa, is a highly efficient vector. Larval crowding and nutrition, as well as the age of the adult, influence the susceptibility of *Culex* to West Nile virus. The virus has been found to infect soft and hard ticks under natural and experimental conditions.

Pathogenesis and Pathology

The pathogenesis of West Nile virus is similar to that of other flaviviruses (180,201,236). West Nile virus is

reported to produce persistent infection and a subacute inflammatory-degenerative process in the CNS of monkeys (255).

Pathologic observations in humans are limited to a very small number of patients with fatal encephalitis and showed lesions of diffuse inflammation and neuronal degeneration. Autopsies performed on patients who died within 4 weeks after inoculation of West Nile virus (used as an experimental treatment for cancer) resulted in West Nile virus isolation from spleen, lymph nodes, liver, and lungs (313), a distribution similar to that described in laboratory animals.

Clinical Features

The incubation period is 1–6 days. The typical case is quite mild, characterized by fever, headache, backache, generalized myalgia, and anorexia. The course of fever may be biphasic. Rash occurs in approximately half of the cases; onset of rash is either during the febrile phase or at the end of it. The rash is roseolar or maculopapular, is nonirritating, and principally involves the chest, back, and upper extremities. Rash may persist for up to a week and resolves without desquamation. Generalized lymphadenopathy is a common finding. Pharyngitis and gastrointestinal symptoms (nausea, vomiting, diarrhea, abdominal pain) may occur. The disease runs its course in 3–6 days, followed by rapid recovery (202). Children generally experience milder illness than adults. Infection may also result in aseptic meningitis or meningoencephalitis in a small proportion of patients, especially in the elderly. In an outbreak of encephalitis in 12 of 49 aged persons who acquired the infection in Israel, four patients died. Three cases of encephalitis in young people were described by Flatau et al. (94); one patient had papillitis. Eleven percent of cancer patients inoculated with the prototype (Egypt 101) strain showed clinical signs of encephalitis (313). There is a single case report of acute anterior myelitis (resembling poliomyelitis) due to West Nile virus infection (116). Other non-neurologic, rare complications include myocarditis (4), pancreatitis (252), and fatal hepatitis (107).

Clinical laboratory findings include leukopenia and, in cases with CNS signs, CSF pleocytosis and elevated protein.

Inapparent and very mild infections are common. In the series of cancer patients intentionally inoculated by Southam and Moore (313), 89% of 78 infected patients had no clinical signs or symptoms other than fever; in 27%, fever never exceeded 1°F.

Diagnosis

On clinical grounds, West Nile fever resembles dengue and other dengue-like fevers. Unlike SLE, JE, and

Murray Valley viruses, West Nile virus can be isolated from the blood of as many as 38% of patients (77% when the specimen is taken on the first day of illness) (109). In persons naturally infected, viremia was detectable up to 5 days after onset; titers were low (maximum titer $\sim 10^{3.3}$ /ml). In cancer patients inoculated with the Egypt 101 strain, viremias were more prolonged and of higher titer (313). Viremia has been demonstrated by chance during the incubation period. The reverse passive hemagglutination test has been used to detect viremia in the blood of nestling birds (101). This technique as well as immunoassays have not been evaluated for detection of virus in human clinical specimens.

Serologic diagnosis is possible using any of the usual tests; cross-reactions with heterologous flaviviruses complicate the interpretation. In addition to standard serologic tests, the indirect IF and ELISA tests are applicable to the diagnosis of West Nile infection. Radial hemolysis-in-gel has been used for the detection of IgG antibodies, has a similar sensitivity to the HI test, and has the advantage that there is no need to remove nonspecific inhibitors (102).

Treatment

Treatment is supportive.

Epidemiology

West Nile virus is widely distributed throughout Africa, the Middle East, parts of Europe and the USSR, India, and Indonesia. During the 1950s, classic studies on the epidemiology of West Nile virus in Egypt and the Sudan revealed that human infections were extremely common in the Nile Delta (325), where 61% of the population acquired the infection by early adulthood. A survey conducted 10–15 years later showed that the infection prevalence had decreased somewhat (to 50%), with the most striking decrease in Cairo (from 60% to 28%) (72). This hyperendemic virus circulation has precluded sharp epidemics but places a high burden of infection on childhood populations, which experience largely unrecognized and clinically undifferentiated febrile disease. This picture may change (especially in areas such as Cairo) as the immune barrier decreases. In a study conducted in 1968, 14.6% of febrile children attending the fever hospital in Alexandria were diagnosed as having West Nile infections (218).

Summertime epidemics of West Nile fever were recognized as early as 1950 in Israel and recurred there at frequent intervals during the 1950s (202). These epidemics involved hundreds of recognized cases, but the true incidence was undoubtedly much higher, and at-

tack rates of over 60% were reported in some localities. The epidemics were the result of amplified virus transmission and spillover to a human population with a low background of immunity.

In an area of South Africa with a relatively low background of immunity to West Nile virus (13–20%), an outbreak in 1974 resulted in infection of 55% of the population. The epidemic involved an area of 2,500 sq km of central and northern Cape Province. Hundreds to thousands of clinical cases occurred, but they were mild and without any recognized cases of encephalitis (212). Few clinical cases have been reported from West and central Africa (107,330). A small outbreak occurred between 1962 and 1964 in the Camargue region of France, in which 13 cases were documented, some with encephalitic complications (249). Infections in tropical Asia are frequent, and the virus appears to be hyperendemic in many areas.

The incidence of CNS infection has not been clearly defined, but this complication appears to be rare. Cases have been described in Israel, India, France, and Egypt. In Egypt, four of 133 patients with aseptic meningitis or encephalitis admitted to one hospital between 1966 and 1968 were shown to have West Nile infection (1).

West Nile virus has been isolated from various mosquito species; *Culex univittatus* and *Culex pipiens molestus* appear to be the most important vectors in Africa and the Middle East (147). The virus has been isolated from *Mansonia metallicus* in Uganda and from *Culex pruina* and *Culex weschei* in the Central African Republic. *Culex tritaeniorhynchus* is an important vector in tropical Asia. There have been numerous isolations from wild birds in many areas, and high antibody rates in birds have been reported in Israel, Egypt, and South Africa. Birds sustain high viremia after experimental infection (210). Mammals, including humans and horses, are incidental hosts and do not play a role in the transmission cycle.

West Nile virus has been isolated from a camel and a grass mouse in Nigeria, ticks in the USSR and Africa, and a frugivorous bat in India (147,170). With the possible exception of the tick isolates, these recoveries are probably of no epidemiologic significance.

Prevention and Control

There is no vaccine. In the event of epidemics, use of space sprays to kill infected adult mosquitoes may be warranted.

Other Viruses

In addition to West Nile and dengue, other flaviviruses cause human disease. These agents are not of

major public health importance but must be considered in the differential diagnosis of febrile illness in persons inhabiting or traveling to endemic areas.

Banji Virus

The Banji virus was first isolated from the blood of a febrile child in South Africa in 1956 (309). It has been associated with human febrile disease in Tanzania and has been isolated from *Culex rubinotus* and other species of mosquitoes, rodents, and sentinel hamsters in Kenya, South Africa, Zimbabwe, and Mozambique. The virus is pathogenic for infant and weaned mice by all routes of inoculation; it causes CPEs in HeLa and primary hamster kidney cells and causes plaques in Vero and LLC-MK2 cells. It has been used as a model to study the pathogenesis of flaviviruses in mice (164).

Bussuquara Virus

Bussuquara virus was originally isolated from a sentinel monkey in Brazil in 1956 (113). The virus is quite distinct from other flaviviruses by neutralization test (48,73). Infant mice are susceptible to intracerebral and intraperitoneal infection, but older mice are only susceptible to intracerebral infection. Adult hamsters inoculated by all routes develop viremia and antibodies but no illness (170). Bussuquara virus replicates and produces plaques in a wide variety of cell cultures, including primary duck and chick embryo, BHK-21, Vero, LLC-MK2, MA-104, and MA-111. Histopathologic lesions resembling yellow fever were found in the sentinel monkey from which the original isolation was made, but subsequent experimental infection failed to reproduce these findings. The virus is known in Brazil and Colombia (26), where it has been isolated from various mosquito species, and in Panama, where it was recovered from the blood of a patient with fever, anorexia, and joint pains lasting 4 days (the only known human case) (316). Antibody prevalence rates of 12% have been found in humans in Panama. The virus has been repeatedly isolated from *Proechimys guyannensis* rodents in Brazil; experimentally inoculated *Proechimys* develop viremia. Mosquitoes of the genus *Culex* are the principal vectors. No control measures are applicable.

Ilheus Virus

Ilheus virus was first isolated from a pool of *Aedes* and *Psorophora* mosquitoes collected in Brazil in 1944. It was originally placed in the West Nile antigenic complex but was shown to be distinct from other members of the complex by neutralization test (48,73). The virus

is pathogenic for infant and weanling mice by the intracerebral and intraperitoneal routes but not for other laboratory animals. It produces plaques in primary rhesus kidney cells and various continuous cell lines (BHK-21, Vero, LLC-in MK2, and porcine kidney) but not in avian cells. Pathologic features are described only in mice, which develop typical encephalitis. A total of eight human infections have been documented by virus isolation—five with mild febrile illness with headache and myalgias, one with encephalitis, and two which were asymptomatic; these occurred in Brazil, Trinidad, Columbia, and Panama (170). The virus was inoculated into 20 cancer patients by Southam and Moore (312); three individuals developed CNS signs. Diagnosis is by virus isolation from blood or serologic tests, which are complicated by cross-reactions with other flaviviruses. The transmission cycle in nature involves wild birds and mosquitoes; at least eight genera of mosquitoes have yielded virus, but most isolations have been made from *Psorophora*. No control measures are applicable.

Kunjin Virus

Kunjin virus was first isolated from *Culex annulirostris* mosquitoes in Australia in 1960. It is a member of the JE antigenic complex and is thus antigenically closely related to Murray Valley encephalitis (MVE) virus. The virus is pathogenic for infant mice by intracerebral and intraperitoneal routes and for weaned mice by intracerebral inoculation. It forms plaques in primary chick embryo, Vero, and porcine kidney cell cultures. Close genetic similarity was shown by restriction digest analysis of cDNA among Kunjin virus strains isolated in widely separated regions of Australia (193). The nucleotide sequence has been obtained and has a very high level of homology with respect to West Nile virus, implying an evolutionary relationship (64). Human disease has been reported from Australia. Two accidental laboratory infections were characterized by mild febrile illness—one with rash and one with lymphadenopathy (6). The first severe case with encephalitis mimicking MVE was described in 1986 (234). However, review of 45 MVE cases during the 1974 epidemic indicated that five patients may have been due to Kunjin virus infection. A case of Kunjin encephalitis has been reported from western Australia. Serologic diagnosis is complicated by cross-reactions with MVE. Kunjin virus was isolated from the spinal cord of a horse with severe encephalomyelitis in New South Wales (13). *Culex annulirostris* is believed to be the principal vector, and wild birds and mammals are viremic hosts. The virus has also been isolated in Sarawak and Thailand.

Rio Bravo Virus

Rio Bravo virus was first isolated from a Mexican free-tailed bat (*Tadarida brasiliensis mexicana*) in California in 1954. Subsequently, many other isolations have been made from bats in California, Texas, New Mexico, and Sonora, Mexico (170). The virus is pathogenic for infant mice. Experimentally inoculated monkeys and hamsters develop infection but no illness. A single naturally acquired human case with febrile illness was reported. Laboratory infection (322) resulted in moderately severe illness in three of five individuals who experienced aseptic meningitis, orchitis, or oophoritis. The virus is not arthropod-borne and does not multiply in inoculated mosquitoes. It is probably transmitted bat to bat by direct contact or aerosol.

Sepik Virus

Sepik virus was isolated from *Mansonia septempunctata* mosquitoes in New Guinea in 1966. Several other isolates have been made from *Ficallbia* and *Armigeres* spp. mosquitoes in New Guinea. Antigenically, Sepik virus is a close relative of Wesselsbron virus. A single human case of febrile illness with headache, requiring hospitalization, has been recorded.

Spondweni Virus

Spondweni virus was originally isolated from *Mansonia uniformis* mosquitoes collected in Northern Zululand, South Africa in 1955 (170). It is ungrouped but is most closely related antigenically to Zika virus. The virus is pathogenic for infant and weanling mice inoculated intracerebrally, produces CPEs in primary hamster and monkey kidney cells, and produces plaques in Vero and LLC-MK2 cells. A virus isolated from the blood of an anicteric child with fever and headache and first thought to be Zika virus was later identified as Spondweni virus; however, recent analysis of this virus strain by monoclonal antibodies suggests that it is a mixture of yellow fever and another flavivirus most closely resembling Spondweni (E. Gould, personal communication, 1988). A volunteer inoculated with this virus developed a mild febrile illness (20). During investigations in South Africa, two persons acquired laboratory infections characterized by fever, chills, aches and pains, nausea, and epistaxis (213). Wolfe et al. (357) reported a case in an expatriate living in Burkino Faso; symptoms were fever, dizziness, nausea, myalgia, headache, photophobia, and a maculopapular pruritic rash. Treatment is symptomatic. Diagnosis is by virus isolation from blood or serology. Spondweni virus has been isolated from at

least seven mosquito species, but most isolates have come from *Aedes circumluteolus* in South Africa. Antibody has been found in cattle, sheep, and goats in South Africa, but the vertebrate hosts involved in transmission are unknown. No control measures are applicable.

Usutu

Usutu virus was first isolated from *Culex naevei* mosquitoes in South Africa in 1959. Newborn and weaned mice are susceptible to fatal encephalitis by the intracerebral route. A single case of human infection with fever and rash was reported from Senegal in 1982. The virus has a wide distribution in sub-Saharan Africa. *Culex* mosquitoes and birds are responsible for transmission in nature.

Wesselsbron Virus

Wesselsbron virus was first isolated from a dead lamb during an epizootic in South Africa in 1955. It is antigenically closely related to Sepik virus and shows some cross-reactivity with yellow fever virus. Wesselsbron immune monkeys develop reduced viremias when challenged with yellow fever virus (158). The virus kills infant and weanling mice by all routes of inoculation. It produces CPE in BHK-21 cells and plaques in Vero and LLC-MK2 cells. Wesselsbron infection in sheep is of veterinary public health importance in southern Africa, where it causes abortion and death of newborn lambs and pregnant ewes (349). A mild, febrile disease occurs in cows. A syndrome of hydrops amnii in pregnant ewes, with prolonged gestation, maternal deaths, and fetal malformation (arthrogryphosis, hydranencephaly, hypoplasia, or segmental aplasia of the spinal cord, neurogenic muscular atrophy, and inferior brachygnathia), has been associated with both wild virus infection and use of a live attenuated vaccine (62). Pathologic changes in lambs include jaundice, focal necrosis of liver cells, Councilman-like bodies, and mild periportal inflammation (187). The clinical features, epidemiology, and pathologic changes in livestock resemble Rift Valley fever, from which it must be distinguished. Naturally acquired human cases of febrile illness occurred in South and West Africa, and there have been several reports of laboratory infections. Human illness is characterized by a short incubation period (2–4 days) and sudden onset of fever, chills, myalgia, hyperesthesia of the skin, hepatosplenomegaly, and maculopapular rash. In severely ill persons, signs of CNS involvement have been noted (211). There have been no fatalities. Clinical laboratory studies show leukopenia and elevated serum transaminase levels. Treatment is supportive. Diagnosis is by virus isolation from blood or

throat swab, or by serology; serologic cross-reactions present difficulties in persons with prior flavivirus infection. The transmission cycle involves mosquitoes. Species incriminated most frequently are *Aedes caballus-juppi*, *Aedes lineatopennis*, and *Aedes circumluteolus* in South Africa and numerous other *Aedes* spp. elsewhere in Africa. The virus is known to occur in Zimbabwe, Cameroun, Nigeria, Senegal, Ivory Coast, Central African Republic, Uganda, Kenya, and Thailand (170). The vertebrate hosts involved in transmission are uncertain; domestic livestock develop high viremias, as do experimentally infected gerbils.

No specific control measures are recommended for prevention of human infection. Special care is required when manipulating this virus in the laboratory. Because of the veterinary hazard, work with the virus in the United States is restricted by the Department of Agriculture.

Zika Virus

Zika virus was first isolated from a sentinel monkey in Uganda in 1947. The virus is not assigned to a subgroup but is most closely related to Spondweni, yellow fever, and Uganda S viruses. Zika immunity suppressed viremia in monkeys challenged with yellow fever (158). The virus kills infant and weanling mice by all routes of inoculation. It produces viremia but no illness in monkeys. Rabbits and guinea pigs inoculated by peripheral routes develop antibodies. Plaque assays may be performed in primary chick or duck embryo cell cultures. Pathologic changes in infected mice include encephalitis, myocarditis, and myositis. Serological surveys reveal a prevalence of human infection of up to 50% in many areas of Africa and in parts of Asia, but human disease has been rarely reported. Of approximately 12 diagnosed human cases, one was in a mosquito collector working in Senegal, 10 were naturally acquired cases that occurred in Nigeria (232) and Indonesia (247), and two were laboratory-acquired. Illness is characterized by fever, malaise, headache, and a maculopapular rash. Treatment is supportive. Diagnosis is by virus isolation from blood or serology; cross-reactions confuse interpretation, especially in persons with prior flavivirus exposures. The virus has been isolated from *Aedes africanus* in Uganda, Ivory Coast, and Central African Republic; *Aedes luteocephalus* in Nigeria and Senegal; *Aedes vittatus*, *Aedes dalzieli*, *Aedes africanus* and *Aedes furcifer-taylori* in Senegal; and *Aedes opok*, *Aedes furcifer*, and *Aedes flavicollis* in Ivory Coast. Although the vertebrate hosts involved in transmission are not defined, it is likely that both nonhuman and human primates play a role and that the transmission cycle is similar to that of yellow fever. Explosive ep-

izootics (without recognized disease) occur in monkey populations in East and West Africa. No control measures are applicable.

FLAVIVIRUSES ASSOCIATED PRIMARILY WITH HEMORRHAGIC FEVER

Yellow Fever

The historical aspects of yellow fever have been reviewed by Strode (320). The disease was recognized as a clinical entity during the seventeenth century in Yucatan. Tropical areas of the Americas were subject to large urban outbreaks in the seventeenth, eighteenth, nineteenth, and early twentieth centuries, and the disease occurred in epidemic foci in the United States as far north as Boston; it also appeared during the eighteenth century in Italy, France, Spain, and England. As late as 1905 there were 5,000 cases and 1,000 deaths in port cities of the southern United States.

Mosquitoes were suggested as the vector of yellow fever by Nott in 1848, but this theory was not seriously proposed until 1881 by Carlos Finlay. In 1900, Major Walter Reed demonstrated a filterable agent in the blood of patients and showed transmission by *Aedes aegypti* mosquitoes. Despite suggestions to the contrary, yellow fever was thought to be transmitted exclusively between human beings by *Aedes aegypti*. Investigations by Soper et al. (311) resulted in the concept of jungle yellow fever, later shown in both tropical America and East Africa to involve wild monkeys and sylvatic mosquito species.

In 1927, Mahaffy and Bauer first isolated the virus by inoculation of a rhesus monkey with the blood of a patient in Ghana; this was the source of the Asibi strain, parent of the 17D vaccine (318). In 1937, Theiler and Smith reported attenuation of the Asibi strain by passage in chick embryo tissue and demonstrated use of the modified virus (17D) for human immunization.

Yellow fever has continued to be a major public health problem in the Americas. Cases are of the jungle type, and no *Aedes aegypti*-borne outbreaks have been reported in nearly 30 years. In Africa, however, large epidemics involving up to 100,000 cases have occurred during the last few decades; both *Aedes aegypti* and various sylvatic vectors have been responsible for transmission.

Infectious Agents

Yellow fever virus is the prototype of the flavivirus genus. It has been used as a model for elucidation of the flavivirus genome structure and replication strategy (see Chapter 25) and for studies on the molecular basis of antigenic structure/function and virulence.

Yellow fever is not placed within an antigenic subgroup or complex by plaque reduction neutralization tests (48,73); however, it is antigenically more closely related to Banzi, Wesselsbron, Bouboui, Zika, and Uganda S viruses than to other flaviviruses. By virtue of its relationship to Zika virus, Spondweni virus may be considered indirectly linked to yellow fever.

Cross-protection between yellow fever and many flaviviruses can be demonstrated using the sensitive intraperitoneal test in infant mice. In more epidemiologically meaningful tests of cross-protection, prior immunization with Wesselsbron, Zika, and dengue viruses caused a significant reduction in viremias of monkeys challenged with virulent yellow fever virus (158,327).

Antigenic differences have been shown between strains of yellow fever virus. By polyclonal antibody absorption techniques, strains from tropical America and Africa are distinct, as are 17D vaccine and parent Asibi viruses (60). Vaccine-specific, vaccine-substrain(17D-204, 17DD)-specific, type-specific, flavivirus-group-reactive, and flavivirus-intercomplex-specific monoclonal antibodies have been defined (296). Monoclonal analyses reveal relationships with viruses belonging to all seven heterologous flavivirus complexes, but yellow fever virus is closer to mosquito-borne viruses than to tick-borne viruses. Some of these relationships (e.g., to Banzi, Uganda S, and Zika) fit previous concepts from polyclonal antibody analyses, but many others do not.

Monoclonal antibodies have not provided an antigenic classification of wild yellow fever strains by geographic origin, and further studies in this direction are required. In one limited analysis, Central and West African strains could be distinguished by immunoprecipitation of NS1 protein by anti-NS1 monoclonal antibodies (79). RNA fingerprinting has delineated four yellow fever topotypes (see section entitled "Epidemiology," below). Strains from South America and Africa are also distinguishable by the electrophoretic migration and carbohydrate content of their E proteins.

Differences have been found between wild yellow fever virus strains in terms of their virulence for mice and monkeys and infectivity for mosquitoes (19,74), but some results are conflicting and reflect multiple variables, including mouse strain, virus passage level, and heterogeneity of virion subpopulations in virus stocks.

Yellow fever virus can be propagated in a wide variety of primary and continuous cell cultures. Vaccine strains (17D and French neurotropic viruses) grow to higher titer and produce more evident CPEs and plaques than do wild strains in various continuous monkey kidney (MA-104, Vero, LLC-MK2), rabbit kidney (MA-111), baby hamster kidney (BHK), and porcine kidney cell lines as well as in primary chick

and duck embryo fibroblast monolayers. Wild yellow fever virus strains can also be propagated in these cell cultures, but plaque formation is inconsistent and variable from strain to strain. Viral growth can be detected by immunofluorescence in advance of the appearance of plaques. Both 17D vaccine and the parent Asibi virus grow in cell cultures of human origin (e.g., Chang liver cells, Henle embryonic intestine, HeLa, KB). Persistent infection of these cells has been described. The virulence properties of virus from carrier cultures can be altered; passage in HeLa cells, in particular, has been associated with a loss of viscerotropism for monkeys (148).

Mosquito cell cultures are useful for primary isolation and are more sensitive than Vero cells or infant mice. *Aedes pseudoscutellaris* (AP-61), cloned *Aedes aegypti*, and *Aedes albopictus* cells are susceptible; infection is generally assessed by immunofluorescence and/or subpassage to mice or Vero cells. AP-61 cells consistently show CPEs. Intrathoracic inoculation of mosquitoes (*Toxorhynchites* or *Aedes aegypti*) is also useful for isolation or assay of yellow fever virus. After a 10- to 16-day incubation period, mosquitoes can be examined directly for virus by immunofluorescence or can be subpassaged to a susceptible host (e.g., suckling mice).

In vertebrate species, yellow fever virus produces both neurotropic and viscerotropic patterns of infection (429). Viscerotropism reflects the pathogenicity of yellow fever virus for human or nonhuman primates infected by the peripheral route; disease is principally characterized by hepatic pathology. Rhesus and cynomolgus macaques, as well as certain neotropical monkeys, are highly susceptible. Monkeys intracerebrally inoculated with wild-type virus develop encephalitis but die of viscerotropic yellow fever.

The European hedgehog (*Erinaceus europaeus*) is the only nonprimate experimental animal which develops severe visceral lesions in response to yellow fever infection. Hamsters intraperitoneally inoculated with some virus strains (e.g., Asibi) are partially susceptible (scattered deaths), but they resist lethal infection with other virus strains. Rabbits resist intracerebral and peripheral challenge but form antibodies. Newly hatched chickens develop viremic infection without disease.

The neurotropic properties of yellow fever virus are most apparent in mice; infant mice are highly susceptible to encephalitis after intraperitoneal or intracerebral inoculation. Considerable variation in neuropathogenicity for mice exists between strains (19). Older mice are susceptible by the intracerebral route, as are guinea pigs.

Pathogenesis and Pathology

Neurotropic yellow fever infection of mice has been used as a model system for studies on the pathogenesis

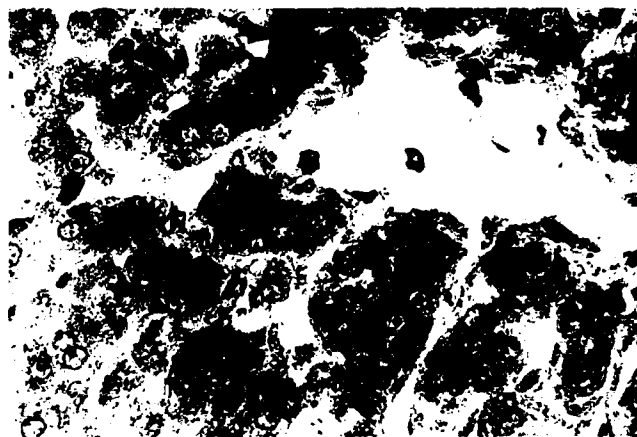


FIG. 11. Postmortem liver sample from a patient with yellow fever, showing midzonal necrosis and hemorrhage, as well as sparing of cells bordering the central vein. Yellow fever viral antigen (arrows) is demonstrated by immunohistochemistry (alkaline phosphatase anti-alkaline phosphatase technique) and is present both in cells undergoing necrotic change and in the cells preserved around the central vein. $\times 500$.

of flavivirus encephalitis (see "Pathogenesis and Pathology," page 771). The discussion to follow deals solely with the viscerotropic disease in humans and nonhuman primate models.

The typical yellow fever lesion is marked by cloudy swelling, then by coagulative necrosis of hepatocytes in the midzone of the liver lobule, sparing cells bordering the central vein (Fig. 11). Eosinophilic degeneration of hepatocytes results in the formation of Councilman bodies and intranuclear eosinophilic granular inclusions (Torres bodies). On an ultrastructural level, Councilman and Torres bodies consist of amorphous material without any viral structures. Multi- and microvacuolar fatty change is nearly always present, especially after the eighth day of illness. An inflammatory response is absent or mild. The reticulin framework is preserved, and healing is complete in recovered cases. Typical changes have been seen in biopsy specimens taken as early as the third day of illness; interpretation of biopsy or necropsy material obtained after the tenth day is often difficult. *Biopsy is contraindicated as a diagnostic procedure due to the high risk of hemorrhage.* In cases in which death is delayed, partially disintegrated Councilman bodies consisting of small, irregular, granular, ochre-colored bodies ("Vilella bodies") may be found, especially in the midzone.

Renal glomerular changes are relatively insignificant compared to acute tubular necrosis and fatty metamorphosis, which may be marked. Schiff-positive transformation of the glomerular basement membrane has been described and has been linked to altered permeability to proteins and albuminuria (221). The myocardial fibers show cloudy swelling, degeneration,

and fatty infiltration. The brain may show edema and petechial hemorrhages. Lymphocytic elements in the spleen and lymph nodes are depleted, and large mononuclear or histiocytic cells accumulate in the splenic follicles (221).

Our present understanding of yellow fever pathogenesis and pathophysiology relies extensively on experimental observations in rhesus monkeys. After inoculation, the virus replicates in regional lymph nodes and then spreads to other tissues, including liver, spleen, bone marrow, and cardiac and skeletal muscle. The hepatic parenchyma is the principal target organ, and hepatocellular damage is undoubtedly mediated directly by viral infection. Serial examination of liver tissue from infected rhesus monkeys reveals the earliest cytopathologic changes and appearance of immunofluorescent viral antigen in Kupffer cells (328). Lymphocytic necrosis in the germinal centers of the splenic periarteriolar lymphocytic sheath and necrosis of germinal centers in lymph node follicles are prominent findings in the rhesus monkey (177,223). Yellow fever (17D) virus replicates to high titer in human peripheral monocyte cultures and in peripheral blood lymphocytes stimulated by phytohemagglutinin (352).

The pathogenesis of the renal lesion is uncertain. In the experimentally infected rhesus monkey, changes in renal function are most revealing during the interval between 36 and 12 hr before death (223). Oliguria was interpreted to reflect intrarenal changes in blood flow secondary to decreased effective blood volume, whereas the development of acute tubular necrosis very late in the infection was the result of the generalized circulatory collapse. Acid-base disturbances, as well as changes in the distribution of water and electrolytes, in cardiac muscle and brain (190) have been described and may be important pathophysiological events.

The pathogenesis of the bleeding diathesis in yellow fever is also complex. Decreased synthesis of vitamin K-dependent coagulation factors by the diseased liver is an important part of the hemorrhagic disorder, but disseminated intravascular coagulation and altered platelet function may play a minor role in severe and fatal cases.

Genetic factors may be important in determining individual host responses to yellow fever infection but have not been well defined. Older reports emphasize the severity of the disease in whites compared to blacks, but it is not possible to separate genetic from acquired resistance factors (e.g., immunity to yellow fever or heterologous flaviviruses). Descendants of Dutch colonists in Surinam who survived typhoid and yellow fever epidemics had gene frequencies that were significantly different from those of a large Dutch control group, possibly indicating selection through genetic control of survival (80).

Clinical Features

The incubation period is usually 3–6 days. The clinical spectrum varies from very mild, nonspecific, febrile illness to a fulminating, sometimes fatal disease with pathognomonic features.

Severe yellow fever begins abruptly with fever, chills, severe headache, lumbosacral pain, generalized myalgia, anorexia, nausea and vomiting, and minor gingival hemorrhages or epistaxes. Despite a persistent or rising temperature, the pulse may decrease (Faget's sign). This syndrome, lasting approximately 3 days, corresponds to the *period of infection*, during which yellow fever virus is present in the blood. It may be followed by a *period of remission*, with defervescence and mitigation of symptoms, usually lasting up to 24 hr. Fever and symptoms reappear with more frequent vomiting, epigastric pain, prostration, and the appearance of jaundice (*period of intoxication*). Viremia is generally absent, and antibodies appear during this phase. The bleeding diathesis is manifested by coffee-ground hematemesis (vomito negro), melena, metrorrhagia, petechiae, ecchymoses, and diffuse oozing from the mucous membranes. Dehydration results from vomiting and increased insensible losses. Renal dysfunction is marked by a sudden increase in albuminuria and diminishing urine output. Death (in 20–50% of severe yellow fever cases) occurs usually on the seventh to tenth day of illness and is preceded by deepening jaundice, hemorrhages, rising pulse, hypotension, oliguria, and azotemia. Hypothermia, agitated delirium, intractable hiccups, hypoglycemia, stupor, and coma are terminal signs. Leukopenia occurs during the acute phase of illness. Other laboratory abnormalities include elevation of bilirubin and serum transaminases, thrombocytopenia, prolonged clotting and prothrombin times, and ST-T wave changes in the electrocardiogram.

Convalescence is sometimes prolonged, with profound aesthenia lasting 1–2 weeks. Late death, occurring at the end of convalescence or even weeks after complete recovery from the acute illness, is a rare phenomenon attributed to cardiac complications or renal failure. The duration of icterus in surviving cases is unknown. Elevations of serum transaminase levels have been documented to persist for at least 2 months after onset of yellow fever (325).

Diagnosis

Mild yellow fever cannot be distinguished clinically from a wide array of other infections. In the presence of jaundice and the other signs of severe yellow fever, conditions that must be differentiated include viral hepatitis, falciparum malaria, leptospiral infections, Rift Valley fever, typhoid, Q fever, typhus, and sur-

gical, drug-induced, and toxic causes. The other viral hemorrhagic fevers, which usually present without jaundice, include dengue, Lassa, Marburg, and Ebola virus diseases, Bolivian and Argentine hemorrhagic fevers, and Congo/Crimean hemorrhagic fever.

Specific diagnosis depends on histopathologic study, isolation of the virus, or demonstration of viral antigen or a specific antibody response. The virus is most readily isolated from serum obtained during the first 4 days of illness, but it may be recovered from serum up to the 14th day and, occasionally, from liver tissue at death. Isolation attempts from clinical specimens can be made by intracerebral inoculation of mice, intrathoracic inoculation of *Toxorhynchites* mosquitoes, or inoculation of mosquito cell cultures. The *Aedes pseudoscutellaris* cell line has the advantage of high sensitivity and a relatively short incubation time (3–6 days) to detection of virus (by immunofluorescence). Viral antigen or IgM-antigen complexes may be detected by immunoassay (227), affording a rapid, early diagnosis. A comparison of various techniques showed that the antigen-capture ELISA has a sensitivity of approximately 70%, and it may detect noninfectious antigen in poorly handled specimens.

Serologic methods useful in the diagnosis of yellow fever include the usual HI, CF, and neutralization tests, single radial hemolysis, indirect immunofluorescence, ELISA, and RIA. The HI, IFA, and neutralization antibodies appear within a week of onset; CF antibodies appear later. The plaque reduction neutralization test (314) has now largely replaced the less sensitive test in mice. Paired acute- and convalescent-phase specimens are required to establish the diagnosis by the rise in antibody titer. Cross-reactions complicate serodiagnosis in cases with prior exposure to heterologous flaviviruses.

A type-specific polypeptide antigen extracted from infected mosquito cell culture membranes appears useful for immunoassays (78). Determination of IgM antibodies by the indirect fluorescent antibody technique or ELISA (189) may indicate recent infection. The duration of IgM antibodies is uncertain, however, and appears to be quite variable. In persons vaccinated with 17D virus, detectable IgM neutralizing antibodies are present as long as 18 months after immunization. IgM antibodies show relative specificity, but cross-reactions in ELISA are sometimes found in patients with prior flavivirus exposures.

The use of yellow fever 17D vaccine may confound serodiagnosis. In persons without prior flavivirus exposure, the vaccine induces a neutralization test seroconversion with a low titer (1:10 to 1:40) of HI antibodies and no detectable IFA or CF antibodies. However, in persons with preexisting flavivirus antibodies, vaccination may result in marked rises in yellow fever and heterologous HI and CF antibodies. The

patterns produced may be broad or heterotypic; in some cases, however, homotypic responses are seen (a specific rise in yellow fever HI or CF antibodies), similar to those seen in recent natural infection.

Treatment

Treatment is supportive. Most patients with yellow fever have not benefited from the availability of modern intensive care, and it is unknown to what extent fluid management and correction of hypotension and electrolyte and acid-base disturbances would reverse the apparently inexorable course of severe yellow fever. A number of compounds with antiviral activity *in vitro* have been described, including ribavirin and derivative compounds (for a review, see ref. 221). Trials of ribavirin in experimentally infected monkeys have yielded conflicting results; the best controlled of these showed no therapeutic effect (C. J. Peters, *personal communication*, 1987). Gamma-interferon treatment of monkeys resulted in delayed onset of viremia and illness but had no effect on survival (10).

Epidemiology

Yellow fever is a zoonotic disease. The primary transmission cycle involves wild nonhuman primates and various sylvatic (tree-hole-breeding) aedine mosquitoes. Humans may be tangentially exposed when they encroach on this cycle (so-called "jungle yellow fever"), and epidemic spread from human-to-human can subsequently be continued by sylvatic vectors. Alternatively, the domestic mosquito, *Aedes aegypti*, which lives in close relationship with humans, may transmit the virus, with humans being the sole viremic hosts in the cycle (*Aedes aegypti*-borne yellow fever or "urban yellow fever").

Yellow fever occurs throughout much of tropical South America and sub-Saharan Africa. Within this region, viral activity may be intermittent and quite localized. The distribution of reported cases gives only a partial picture of the natural circulation of yellow fever virus and gives a misleading estimate of risk to travelers (Fig. 12).

The annual incidence of officially reported yellow fever cases is 50–300 cases in tropical America and 5–1,000 cases in Africa. These data represent a significant underestimate of the true morbidity, as shown by investigations of various epidemics (Table 4).

In tropical America the incidence of jungle yellow fever is highest during months with peak rainfall, humidity, and temperature (January to March). In Africa, transmission by *Aedes aegypti* and tree-hole-breeding mosquitoes peaks during the late rainy season and early dry season. In tropical America, jungle yellow

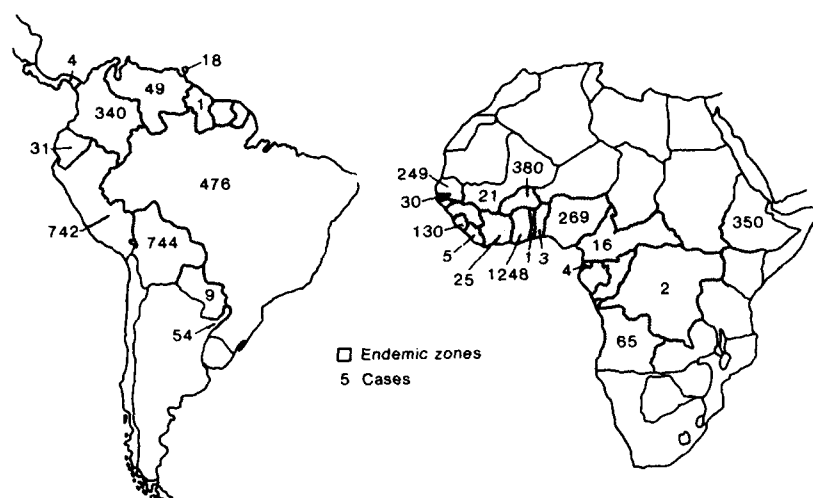


FIG. 12. Yellow fever endemic zone (shaded) and the number of cases officially reported to the World Health Organization, by country, 1965–1985.

fever principally affects young adult males. The age/sex distribution reflects the higher incidence of exposure to *Haemagogus* vectors during wood-cutting and forest-clearing activities in the forest. In Africa, background immunity (natural and vaccine-induced) is the principal factor determining the age distribution of cases. In outbreaks affecting immunologically virgin populations (e.g., in Ethiopia, 1960–1962), all ages are equally affected. In West Africa, a high level of acquired immunity in adults has resulted in high attack rates in children. The ratio of inapparent to apparent yellow fever infection is ~10-fold higher in individuals sustaining primary yellow fever infection than in persons with prior heterologous flavivirus immunity (224).

Yellow fever has never occurred in Asia. Possible explanations include (a) cross-protection afforded by dengue immunity and (b) low vector competence of Asian strains of *Aedes aegypti*; experimental evidence in support of both hypotheses has been presented (for a review, see ref. 220).

TABLE 4. Yellow fever cases and deaths officially reported to the WHO and estimates of morbidity and mortality from direct investigations of epidemics

Country	Year(s)	No. of cases (deaths)	
		Officially notified	Determined by epidemiologic investigation
Ethiopia	1960–1962	— ^a (3,000)	100,000 (30,000)
Senegal	1965	243 (216)	2,000–20,000 (200–2,000)
Burkina Faso	1969	87 (44)	3,000 (100)
Nigeria	1969	208 (60)	100,000 (— ^a)
Nigeria	1970	4 (1)	786 (15–40)
Gambia	1978–1979	30 (30)	5,000–8,000 (1,000–1,700)
Nigeria	1986	3,291 (623)	9,800 (5,600)

^a No estimate available.

Ecology of Yellow Fever in Tropical America

Howler monkeys (*Alouatta* sp.), spider monkeys (*Ateles* sp.), squirrel monkeys (*Saimiri* sp.), and owl monkeys (*Aotus* sp.) are effective viremic hosts and commonly develop fatal infections, whereas capuchin monkeys (*Cebus* sp.) and woolly monkeys (*Lagothrix* sp.) are susceptible to viremic infection but usually do not develop clinical signs. This unstable host-parasite relationship may reflect the relatively recent introduction of the virus, possibly at the time navigation was established between Africa and America during the fifteenth century. Other South American vertebrates, including edentates, marsupials, and rodents, are now believed to play a negligible role in the yellow fever transmission cycle, although further study appears warranted.

Mosquitoes of the genus *Haemagogus* are the principal vectors of jungle yellow fever in tropical America. *Haemagogus* breed in tree holes and feed in the forest canopy during the midday hours, but they also have been found biting humans in forest clearings and even inside houses in villages near the forest. Transovarial transmission of yellow fever virus in *Haemagogus* has been experimentally demonstrated (86). This phenomenon may explain, in part, maintenance of the virus during prolonged dry seasons, when adult vector populations are diminished. The relatively drought-resistant mosquito *Sabethes chloropterus*, a relatively inefficient vector, may also play a role in virus survival.

The development of anti-*aegypti* campaigns in Latin America during the twentieth century culminated in eradication of the vector from most countries surrounding the Amazon Basin and the disappearance of urban yellow fever after 1942. However, within the last decade, *Aedes aegypti* has reinvaded many areas, in

juxtaposition with the jungle yellow fever cycle, raising the specter of future urban outbreaks.

Ecology of Yellow Fever in Africa

All species of cercopithecoid and colobid monkeys tested have proved to be effective viremic hosts, circulating virus for several days or more at sufficient titers to infect mosquitoes. Infection infrequently results in illness or death, indicating a balanced host-parasite relationship.

Aedes africanus is responsible for year-round virus transmission in the humid equatorial African forests (Fig. 13). In zones bordering high forested areas of East Africa, *Aedes bromeliae* (formerly known as *Aedes simpsoni*) links the forest cycle with humans and is responsible for intense interhuman spread during epidemics.

The ecologic zones bordering equatorial forest in West and Central Africa have assumed great importance in yellow fever ecology. Appropriately named the "zone of emergence" by Germain et al. (108), the savannah vegetational zones support large and concentrated populations of monkeys and vector mosquitoes. Viral activity intensifies during the rainy season and wanes during the dry season, when vector populations virtually disappear. The principal species involved in sylvatic transmission and transmission to humans are *Aedes fuscifer*, *Aedes africanus*, and *Aedes luteocephalus*. These species are responsible for interhuman spread during epidemics. Other vectors which play a secondary or accessory role in yellow fever transmission cycles include *Aedes vittatus*, *Aedes metallicus*, *Aedes opok*, and *Aedes neoafricanus*.

In areas subject to extreme drying (e.g., in the dry

northern Sudan and Sahel savannah zones of West Africa), yellow fever occurs in intermittent epidemic form, and human immunity patterns indicate little or no infection during interepidemic periods. In these areas, domestic water storage is intensively practiced, domestic *Aedes aegypti* populations are high, and introduction of yellow fever virus may result in explosive outbreaks. Urban areas along the West African coast are also susceptible. A large urban epidemic occurred in Nigeria in 1987. Vertical transmission of yellow fever virus has been documented experimentally in *Aedes aegypti* (3). Evidence for vertical transmission in nature has been obtained by virus isolation from male *Aedes fuscifer* in West Africa (66). This mechanism ensures virus survival over the long dry season. Yellow fever virus has been isolated from *Amblyomma variegatum* ticks in the Central African Republic (292), raising the possibility that alternate vectors may play a role in dispersal or dry-season maintenance of the virus.

Yellow fever virus has been rarely isolated from other arthropods, including *Aedes dentatus*, *Coquilletidia fuscipennata*, and phlebotomine flies. Virus has been isolated from a bat in Ethiopia. These observations are of interest but probably bear little relationship to the ecology of yellow fever.

Molecular Approaches

RNA oligonucleotide fingerprinting has distinguished at least four geographic topotypes [one in South America and three in Africa (75)], suggesting separation and evolutionary change. Within a single region, however, evolution appears to be quite slow, as shown by genetic homogeneity among strains belonging to the Senegalese topotype.

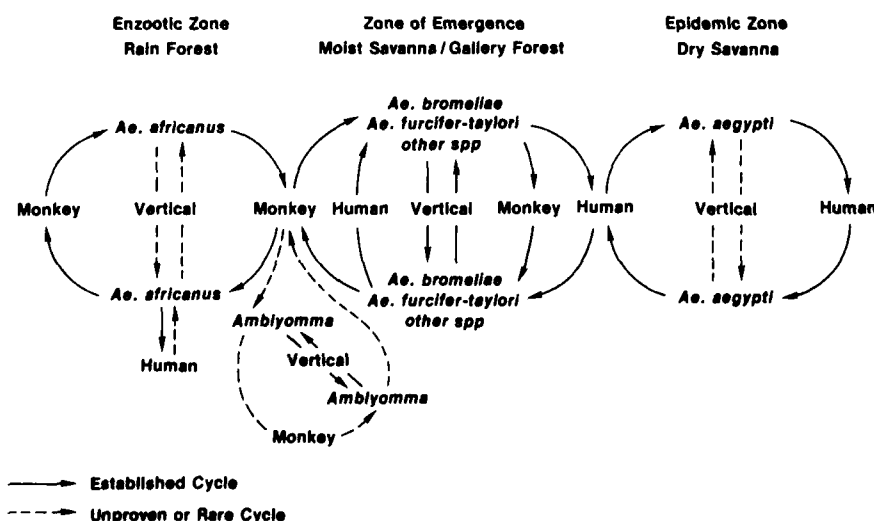


FIG. 13. Complex ecological relationships of yellow fever virus in Africa. In the rain forest zone of Central and West Africa, the virus is maintained in a continuous cycle of low-level transmission, involving monkeys and *Aedes africanus* mosquitoes. In savanna areas with gallery forest along water-courses, high rates of virus transmission to both monkeys and humans may occur during the rainy season, fueled by high densities of tree-hole-breeding *Aedes* vectors. In the very dry savanna areas, where water storage is practiced by the population, domestic *Aedes aegypti* may be responsible for interhuman spread if the virus is introduced from the endemic zones.

Prevention and Control

Vaccination

Yellow fever 17D is a safe, effective live viral vaccine prepared from infected chicken embryos under standards developed by the World Health Organization (WHO) (308). Demonstrable immunity occurs in over 95% of vaccinees within 10 days. For the purposes of international certification, immunization is valid for 10 years, but various studies have shown persistence of antibodies for as long as 30–35 years; immunity is probably lifelong (258). Serious adverse reactions to 17D vaccine are extremely uncommon. No abnormalities in liver function tests are associated with 17D vaccination. Fewer than 10% of vaccinees experience headache and malaise. Allergic reactions occur at a very low rate (approximately one in a million), predominantly in persons with an allergy to eggs. Neurological accidents are extremely uncommon and have been limited to infants (9). The vaccine should not be given to children under 6 months of age or to pregnant women. Persons with known immunodeficiency states (including clinically overt HIV infection) or on immunosuppressive drugs should also not receive yellow fever vaccine.

Vaccination results in a low-level viremia lasting 1–2 days and beginning 3–4 days after inoculation. The low magnitude of viremia and the fact that *Aedes aegypti* is refractory to oral infection with 17D virus preclude the possibility of natural transmission (and possible reversion) of vaccine virus.

Factors that may affect seroconversion to the vaccine include: (a) nutritional state; (b) simultaneous administration of other vaccines; and (c) preexisting heterologous flavivirus immunity. Children with kwashiorkor show marked impairment in antibody production after 17D vaccination. Persons administered 17D yellow fever and cholera vaccines simultaneously or 1–3 weeks apart showed reduced antibody responses to both vaccines (105). Other vaccine combinations can be used without interference. Studies with 17D vaccine produced in mouse brain and administered by scarification have shown a reduction in vaccine seroconversion in African population groups with prevaccination heterologous flavivirus antibodies. In persons given 17D chick embryo vaccine by the subcutaneous route, however, preexisting heterologous immunity did not interfere with the immune response.

At the present time, vaccines produced by some of the world's 12 manufacturing institutes are contaminated with avian leukosis virus. Although undesirable, this contaminant has not been associated with the development of leukemia, lymphoma, or other cancers (343).

The French neurotropic vaccine, produced from infected suckling mouse brains, is no longer manufactured. The vaccine had the advantage of high stability and ease of administration (by scarification or multiple puncture). However, approximately 20% of vaccinees developed systemic symptoms, 3–4% developed meningeal signs, and 0.5–1.3% developed postvaccinal encephalitis. Neurologic accidents were more frequent in children than in adults; fatalities and permanent neurologic sequelae have been reported.

Other Preventive Measures

Areas infested with the domestic form of *Aedes aegypti* are at risk of the introduction and urbanization of yellow fever. Elimination of breeding sites (tires, artificial containers, etc.), treatment of potable water with temephos (Abate), perifocal spraying with organophosphorus insecticides, and use of *Gambusia* minnows are effective if applied in a well-administered and continually supported program.

In the case of an outbreak of *Aedes aegypti*-borne yellow fever, ground or aerial ultra-low-volume (ULV) application of adulticides may be used. The control of yellow fever epidemics involving wild vector species is more difficult, and little experience with vector control has been accumulated.

Aerial ULV applications of malathion were shown to be effective for the control of the yellow fever vector *Aedes bromeliae* breeding in false banana (*Musa ensatta*) plantations in Ethiopia (38). Ground and aerial applications of malathion rapidly suppressed populations of *Aedes africanus* in forest habitats in West Africa for a period of time believed sufficient to interrupt virus transmission (17). Aerial ULV was also used for the control of *Haemagogus* vectors in forested areas in eastern Panama in 1974.

Dengue Hemorrhagic Fever

Cases of hemorrhage and death had been described during outbreaks of classic dengue fever in Australia (in 1897), Greece (1928), and Formosa (1931) (166). In 1954 a febrile disease with hemorrhagic signs (Philippine hemorrhagic fever) occurred in epidemic form in Manila and was shown to be caused by dengue types 3 and 4. Dengue hemorrhagic fever was subsequently described in many other areas of Southeast Asia, where it is now an important cause of endemic and epidemic morbidity. Sporadic cases were recognized in the Caribbean during the 1970s, and in 1981 a large outbreak occurred in Cuba. Halstead proposed that the severe form of the disease had an immunopathologic

basis and occurred in individuals previously sensitized by infection with a heterologous dengue serotype (134–136).

Infectious Agents

All four dengue virus serotypes cause dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). In Thailand, dengue type 2 was the predominant virus associated with these syndromes until the 1980s, when dengue types 3 and 4 emerged as important serotypes. In Indonesia, dengue type 3 was most frequently isolated and associated with fatal cases between 1976 and 1978. The sequence of infecting serotypes, the interval between infections, and strain differences in virulence may be important determinants of the clinical and epidemiological patterns of DHF (124,134). Since laboratory markers of virulence have not been developed, this question has eluded study. Recent investigations indicate that viral growth in mononuclear cells may provide a correlate of virulence. The considerable degree of heterogeneity of the dengue virus genome demonstrated by RNA mapping (see section entitled "Dengue Fever," above) illustrates the potential for significant biologic variations.

Pathogenesis and Pathology

There is no satisfactory animal model of DHF. Pathologic findings in fatal human cases have been reviewed by Bhamarapravati et al. (24), Burke (47), and Reyes (270). Gross pathological findings include: petechiae, ecchymoses, and focal visceral hemorrhages; serous and bloody effusions; retroperitoneal edema; and, in some cases, hepatic enlargement. On histopathologic examination, there are diffuse small-vessel changes in viscera and soft tissues with leakage of plasma (perivascular edema) and erythrocytes. The spleen and lymph nodes show proliferation of immature and mature lymphoid cells and plasmacytoid elements. Necrosis of thymus-dependent areas of the spleen may be prominent. Hepatic lesions similar to other hemorrhagic fevers, though generally less severe, consist of central or paracentral focal necrosis, sinusoidal acidophilic (Councilman) bodies, hypertrophy of Kupffer cells, variable and mild fatty change, and patchy portal mononuclear cell infiltration. Bone marrow changes include maturational arrest of megakaryocytes. Focal dengue viral antigen has been demonstrated in skin, liver, and mononuclear leukocytes.

Prospective studies in Thailand have shown that DHF is principally a disease of children who have previously been infected with at least one heterologous dengue serotype (291). Children with secondary infections were at significantly higher risk of developing

DHF than those experiencing their first infection. The concept of antibody-dependent immune enhancement of dengue viral replication in monocytes has been proposed to explain this observation (see section entitled "Antibody-Dependent Enhancement," page 768). Attempts to model this phenomenon *in vivo* by studying viremia levels in immunologically sensitized nonhuman primates (133) have provided equivocal results (280).

Increased replication of dengue virus in mononuclear leukocytes may be associated with a secondary set of reactions in the host's attempt to eliminate dengue-infected cells, resulting in release of vasoactive mediators of shock and procoagulants.

Bradykinin has not been implicated (88). Increased vascular permeability results in hemoconcentration, decreased effective blood volume, tissue hypoxia, lactic acidosis, and shock. The etiology of the hemorrhagic disturbance in DHF appears to be complex. Microvascular injury, thrombocytopenia, platelet dysfunction, and disseminated intravascular coagulopathy have been variously implicated. Increased platelet turnover has been attributed to the direct attachment of dengue virus to platelets and to the presence of antiplatelet antibodies, with subsequent immune elimination.

The reasons for expression of severe disease in a small subset (2–6%) of persons with dengue sequential infections remains controversial. Both virus-specified and host-related factors may operate. Virus strains may vary with respect to the presence of enhancing epitopes or other virulence factors. Macrophages activated with bacterial cell wall products and peptidoglycans show enhanced virus replication, suggesting that coincident infections could influence dengue pathogenesis.

Clinical Features

The clinical manifestations of DHF and DSS are described by Cohen and Halstead (63) and by Nimmanitya et al. (239). The disease initially presents in a manner similar to that of classic dengue fever (see section entitled "Dengue Fever," above) but progresses after 2–5 days to a rapidly progressive severe form with prostration, restlessness, irritability, shock with cold clammy extremities, diaphoresis, circumoral and peripheral cyanosis, rapid respiration, rapid pulse, and hypotension. Spontaneous hemorrhages occur, including petechiae, ecchymoses, oozing from venipuncture sites, epistaxis, etc. Gross hematuria and gastrointestinal hemorrhages and intracerebral bleeding are relatively rare but may be life-threatening. Physical findings include skin hemorrhages, pleural effusions, changes in vital signs, and hepatomegaly. Laboratory

abnormalities include elevated hematocrit, thrombocytopenia, hypoproteinemia, depression of complement (especially C3) and fibrinogen levels, and the presence of fibrin split products in plasma. The progression of shock is rapid; without physiologic treatment, up to 50% of patients with severe disease die. However, early recognition and appropriate treatment have resulted in case-fatality rates of under 1%.

Diagnosis

Geographic location and epidemiologic setting are important clues to the diagnosis. DHF has not occurred in areas affected by other viral hemorrhagic fevers. Chikungunya (see Chapter 26) occasionally produces an illness with minor hemorrhagic manifestations similar to those of classic dengue fever, but cases with severe hemorrhage, hemoconcentration, thrombocytopenia, and shock are very rare or do not occur. Nonviral causes must be considered in the individual case, including bacterial sepsis, scrub and epidemic typhus, leptospirosis, severe malaria, and typhoid fever.

Virus isolation from tissues of fatal cases is less often successful than in classic dengue (241). Techniques for virus isolation from blood of acutely ill patients are discussed in the section entitled "Dengue Fever," above. Serodiagnosis is possible, but identification of the infecting subtype is difficult because most cases of DHF occur in persons with prior dengue exposure. Anamnestic antibody responses are characterized by high HI and CF antibody titers, which are broadly cross-reactive. In some cases, neutralization tests demonstrate the phenomenon of original antigenic sin; if the current infection is known by virus isolation, the sequence of infection may thus be revealed.

Treatment

The World Health Organization (361) has formulated specific guidelines for the management of cases. Principles of treatment are dictated by the need to closely monitor the patient's vital signs and hematocrit and to replace plasma volume by judicious fluid replacement. Oxygen should be administered; moreover, if disseminated intravascular clotting is documented, consideration may be given to heparin therapy. Blood transfusion is indicated only in the case of severe hemorrhage. Salicylates and hepatotoxic drugs should be avoided. Corticosteroids are widely used; evidence for their usefulness is conflicting, but some studies indicate that they are of no value (323).

Specific, antiviral therapy has not been extensively evaluated. An uncontrolled trial of interferon was con-

ducted during the 1981 epidemic in Cuba, with some indication that deaths may have been averted.

Epidemiology

Dengue hemorrhagic fever is a leading cause of morbidity and mortality in tropical Asia, where it is endemically established. In Thailand in 1977, for example, DHF was the second leading cause of death due to infectious disease. During the past 30 years, over 700,000 cases of DHF have been officially reported, with major epidemics in the People's Republic of China, the Socialist Republic of Viet Nam, Indonesia, Thailand, and Cuba—and over 20,000 deaths (135). In the Cuban outbreak of 1981, 344,203 persons acquired clinical dengue, of whom ~10,000 had hemorrhagic fever and 158 died (1.6%). Although individual patients with DHF had been reported previously in the Caribbean, this was the first epidemic in the region.

Dengue hemorrhagic fever in Asia is a disease of childhood. Two peaks have been noted in age-specific incidence rates: children under 1 year old and children 3–5 years of age. The disease in infants is associated with primary infection in the presence of maternal antibody, whereas the vast majority of cases in older children is the result of secondary infections. Studies in Thailand have estimated the frequency of shock syndrome to be 11 cases per 1,000 secondary dengue infections (44). An age-dependent excess in cases of severe DHF with shock syndrome in girls, compared with boys, has been noted and appears to be related to host factors, since serosurveys have shown no difference in sex-specific antibody prevalence (134). Other risk factors include (a) race (whites were significantly more frequently affected than blacks in Cuba) and (b) underlying chronic diseases such as sickle cell disease, diabetes mellitus, and bronchial asthma (32).

Prevention and Control

See section entitled "Dengue Fever," above.

Other Flaviviruses Associated with Hemorrhagic Fever

Kyasanur Forest Disease

Kyasanur Forest virus was isolated from a sick monkey (*Presbytis entellus*) in the Kyasanur Forest, Shimoga District, Karnataka (then Mysore) State, India in 1957 (359). The virus belongs to the TBE antigenic complex. No antigenic differences between strains have been found. The virus is lethal to infant and wean-

ling mice by both the intracerebral and intraperitoneal routes, produces CPEs or plaques in chick embryo, hamster, and monkey kidney cell cultures, and replicates without CPEs in a continuous cell line of *Haemaphysalis spinigera* tick cells. Pathologic findings in human patients include parenchymal degeneration of the liver and kidneys, hemorrhagic pneumonitis, and an increase in reticuloendothelial tissue in liver and spleen, with marked erythrophagocytosis (198). Similar changes were seen in experimentally infected monkeys, which also showed encephalitic lesions (chromatolysis of neurons and focal demyelination) (345). Lactating monkeys shed small amounts of virus in their milk. The virus persisted in tissues of mice which survived acute infection with sequelae of frank paralysis (259). The clinical illness in humans is characterized by fever, headache, myalgia, cough, bradycardia, dehydration, hypotension, gastrointestinal symptoms, and hemorrhages. In some patients a biphasic course is seen; the first phase, as described above, lasts 6–11 days, followed by an afebrile period of 9–21 days and then the reappearance of fever and signs of meningoencephalitis. In such patients, the disease closely resembles Central European TBE (347). Leukopenia is a frequent finding during the acute phase of illness, and serum transaminase levels are raised. Diagnosis is by virus isolation from blood or serology. Standard serological tests and enzyme-linked immunoassays are applicable. Viremia is detected between the second and twelfth days of illness and is maximum between the third and sixth days, with a mean titer of 3.0 dex. The disease is limited to Mysore State, India but is gradually spreading. Epizootics occur in wild monkeys. Human infections occur principally during the dry season and in persons with close contact with forested areas. Thousands of cases have been reported since the recognition of the disease in 1957; the annual incidence of virologically diagnosed cases varies between 400 and 500 cases. The case-fatality rate is 3–5%; no sequelae are reported. The basic transmission cycle involves ixodid ticks and wild vertebrates, principally rodents and insectivores. Bats and ground-dwelling birds may play a role in transmission. Large animals (goats, cows, sheep) become infected, but viremias are low and their importance is principally as hosts sustaining tick populations. At least 10 species of ixodid ticks have been implicated in transmission; *Haemaphysalis spinigera* is a major vector (16). Transstadial and transovarial transmissions occur in ticks. The density of tick vectors in a given year correlates with the incidence of human disease. A considerable body of research is available on the use of a killed vaccine; although serologic responses are rather weak, the vaccine has been shown to confer protection in field trials (16).

Omsk Hemorrhagic Fever

Omsk hemorrhagic fever virus was first isolated in 1947 from the blood of a patient with hemorrhagic fever during an epidemic in Omsk and Novosibirsk Oblasts, USSR. The agent is a member of the TBE virus complex. Strain heterogeneity has been noted in virulence and antigenic characteristics. The virus is pathogenic for infant and weanling mice by all routes of inoculation. Guinea pigs inoculated by the subcutaneous route develop fever and scattered deaths; rabbits develop antibodies only. The virus causes hemorrhagic disease and death in experimentally inoculated muskrats and narrow-skulled voles (*Microtus gregalis*). The virus has been propagated in pig kidney, Hela, and Detroit G cells. The disease in humans closely resembles Kyasanur Forest disease except that sequelae (hearing loss, hair loss, neuropsychiatric complaints) are relatively frequent (438). The case-fatality rate is 0.5–3%. Between 1945 and 1958 a total of nearly 1,500 cases were recorded in the Omsk region. Small numbers of cases occurred in the 1960s, but in recent years there have been no official notifications. The disease affected rural populations engaged in field work during the spring and summer. Muskrat hunters were at highest risk.

Muskrats were imported into the lake district of western Siberia from North America during the early 1900s, are susceptible to lethal infection, and experience epizootic die-offs. The basic transmission cycle remains uncertain. The ixodid tick vector *Dermacentor reticulatus* has been incriminated by field evidence and experimental infection. Other species, especially *Ixodes apronophorus*, are suspected to play a role in virus maintenance. Rodents, particularly water voles (*Arvicola terrestris*), are the principal viremic hosts. Direct rodent-to-rodent transmission may occur. Muskrats are epizootic hosts, and human infections occur by direct contact with urine, feces, or blood. Virus isolations have also been made from several mosquito species, from a gamasid mite, and from sentinel mice. No specific Omsk hemorrhagic fever vaccine has been developed, but TBE vaccines apparently provide cross-protective immunity and have been used in high-risk population groups.

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